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DISORDERS OF CROSS-TALK BETWEEN NUCLEAR AND MITOCHONDRIAL GENOMES: MOLECULAR APPROACH AND STRATEGIES TO A THERAPY

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LIST OF PUBLICATIONS

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IV. Nogueira C, Barros J, Sá MJ, Azevedo L, Taipa R, Torraco A, Meschini MC, Verrigni D, Nesti C, Rizza T, Teixeira J, Carrozzo R, Pires MM, Vilarinho L, Santorelli FM, Novel *TTC19* mutation in a family with severe psychiatric manifestations and complex III deficiency. Neurogenetics. 2013 May;14(2):153-160.

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VI. Nogueira C, Meschini MC, Nesti C, Garcia P, Diogo L, Valongo C, Costa R, Videira A, Vilarinho L, Santorelli FM, A novel *SUCLA2* mutation in a Portuguese child associated with “mild” methylmalonic aciduria. J Child Neurol. 2013 *In press*

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ABBREVIATION INDEX

2D	Two-dimensional
ad	Autosomic dominant
ADP	Adenosine diphosphate
AHS	Alpers Huttenlocher syndrome
AHSCT	Allogeneic haematopoetic stem-cell transplantation
AICAR	5-Aminoimidazole-4-carboxamide ribotide
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANT	Adenine nucleotide translocator
ar	Autosomic recessive
ARSAL	Autosomal recessive spastic ataxia with leukoencephalopathy
ARSs	Aminoacyl-tRNA synthetases
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BN-PAGE	Blue native polyacrylamide gel electrophoresis
bp	Base pair
BTHS	Barth syndrome
C4DC	C4-dicarboxylic carnitine or succinylcarnitine
Ca^{2+}	Calcium
CHKB	Choline kinase-β
CK	Creatine kinase
CMT	Charcot-Marie-Tooth
CNS	Central nervous system
CoQ10	Coenzyme Q10
COX	Cytochrome oxidase
CSF	Cerebral spinal fluid
Ct	Cycle threshold
dAMP	Deoxyadenosine monophosphate
dGK	Deoxyguanosine kinase
dGMP	Deoxyguanosine monophosphate
D-loop	Mitochondrial non-coding region
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTPS	Deoxynucleotide triphosphates
EF	Elongation factors

ERRs	Oestrogen-related receptors
$FADH_2$	Flavine adenine dinucleotide
FOXO	Forkhead box O
GTP	Guanosine-5'-triphosphate
H	Heavy
H_2O_2	Hydrogen peroxide
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP	Heavy strand promoter
HUPRA	Hyperuricemia, pulmonary hypertension, renal failure and alkalosis
IMM	Inner mitochondrial membrane
kDa	Kilo Dalton
KSS	Kearn-Sayre syndrome
L	Light
LBSL	Leukoencephalopathy with brainstem and spinal cord involvement and high lactate
LHON	Leber hereditary optic neuropathy
LRPPRC	Leucine-rich pentatricopeptide repeat containing protein
LSFC	French–Canadian variant of Leigh syndrome
LTBL	Leukoencephalopathy with thalamus and brainstem involvement and high lactate
MAM	Mitochondria-associated membrane
MCU	Mitochondrial calcium uniporter
MDS	MtDNA depletion syndrome
MEGDEL	Megaconial encephalomyopathy, 3-methylglutaconic aciduria, deafness, and Leigh syndrome-like encephalopathy
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
MERRF	Myoclonic epilepsy and ragged red fibres
mETC	Mitochondrial electron transport chain
Mfn	Mitofusin
MILS	Mitochondrial inherited Leigh syndrome
miRNA	MicroRNA
MLASA	Mitochondrial myopathy and sideroblastic anemia
MLPA	Multiplex ligation-dependent probe amplification
MMA	Methylmalonic acid
MNGIE	Mitochondrial neurogastroIntestinal encephalomyopathy
MnSOD	Manganese superoxide dismutase
MRC	Mitochondrial respiratory chain

MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRP	Mitochondrial ribosomal protein
mtDNA	Mitochondrial deoxyribonucleic acid
mtEF	Mitochondrial elongation factor
mTERF	Mitochondrial termination factor
mtIF	Mitochondrial initiation factor
mTOR	Mammalian target of rapamycin
mtRF	Mitochondrial release factor
MTS	Mitochondrial targeting sequences
mt-tRNA	Mitochondrial transfer ribonucleic acid
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NARP	Neuropathy ataxia and retinitis pigmentosa
nDNA	Nuclear deoxyribonucleic acid
NRF1	Nuclear respiratory factor 1
O_2	Oxygen
O_2^-	Superoxide anion
$^{\cdot}OH$	Hydroxyl radical
OMIM	Online Mendelian Inheritance in Man
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PARPs	Poly ADP ribose polymerases
PBS	Phosphate buffered saline
PCHD-6	Pontocerebellar hypoplasia type 6
PCR	Polymerase chain reaction
PEO	Progressive external ophthalmoplegia
PGC1α	Proliferator-activated receptor gamma coactivator 1-alpha
POLG	Polymerase gamma
POLRMT	Mitochondrial RNA polymerase
PPAR	Peroxisome proliferator-activated receptor
PS	Pearson syndrome
PUSI	Pseudouridine synthase I
qPCR	Real-time quantitative PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RRFs	Red ragged fibers
rRNA	Ribosomal ribonucleic acid
RXRα	Retinoid X receptor-α
SANDO	Sensory ataxic neuropathy, dysarthria and ophthalmoparesis
SCAE	Spinocerebellar ataxia – epilepsy syndrome
SCS	Succinyl-CoA synthetase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIRT1	Sirtuin 1
SNP	Single nucleotide polymorphism
STACs	Sirtuin-activating compounds
TACO1	Translational activator of COX I
TBS	Tris buffered saline
TCA	Tricarboxylic acid
TEFM	Transcription elongation factor mitochondrial
TFAM	Mitochondrial transcription factor A
TFB1M	Mitochondrial transcription factor B1
TFB2M	Mitochondrial transcription factor B2
TIM	Translocase of the inner membrane
TOM	Translocase of the outer membrane
TOP	Mitochondrial topoisomerases
TP	Thymidine phosphorylase
TRMU	tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase
tRNA	Transfer ribonucleic acid
T-TBS	Tween Tris Buffered Saline
UDP	Uniparental dissomy
UQCR	Ubiquinone–cytochrome *c* reductase
WT	Wild-Type

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ABSTRACT

Mitochondrial dysfunction accounts for an important and heterogeneous group of inherited metabolic disorders with hitherto no effective therapeutic options. Most of the known mitochondrial disorders are caused primarily by a dysfunctional oxidative phosphorylation system (OXPHOS) and consequently a deficient energy production. OXPHOS depends on the coordinated expression of both nuclear and mitochondrial genomes. Therefore, mitochondrial diseases can be caused by genetic defects in the mitochondrial or the nuclear genome. This may affect the interplay between the two genomes, causing nuclear-mitochondrial intergenomic communication disorders. The organelle/nucleus cross-talk has gained increased relevance in the past years and since then many genes have been identified as being involved in these diseases. Mitochondrial cross-talk disorders are particularly severe and can be divided in two groups: i) mitochondrial DNA (mtDNA) multiple deletions syndrome, that share features such as ocular and limb myopathy, and ii) mtDNA depletion syndrome (MDS) which comprises a heterogeneous group of autosomal recessive disorders occurring in infancy or early childhood with three clinical categories: myopathic, encephalomyopathic and hepatocerebral MDS.

In this work 47 patients clinically suspicious of intergenomic communication disorders were selected to be investigated at the molecular level. In 55% of these patients mtDNA depletion or multiple deletions were present. From these, “primary” mtDNA depletion was detected in 17 patients, whereas “secondary” mtDNA depletion was identified in eight cases and mtDNA multiple deletions were shown in only one patient. The spectrum of identified mutations encompasses the following genes: *MPV17*, *DGUOK*, *SUCLA2*, *C10orf2*, *POLG*, and *TAZ,* in patients with “primary” mtDNA depletion; *TTC19* and *RARS2* in those with “secondary” mtDNA depletion; and *TYMP* in a patient with mtDNA multiple deletions. However, some of the studied patients still remain undefined, because no mutations were detected, demonstrating that whole exome sequencing could be an effective alternative to seek for new candidate genes. Additionaly, we tested some therapeutical agents *in vitro* in order to appreciate their efficacy in the enhancement of mitochondrial ATP generation and in the reduction of ROS production. These potential therapeutic compounds can have an impact on patients health and offer novel prospective against mitochondrial diseases.

RESUMO

As citopatias mitocondriais constituem um importante grupo de doenças hereditárias do metabolismo de expressão clínica heterogénea, para as quais não existe uma terapia eficaz. A maioria das doenças mitocondriais descritas são causadas por uma disfunção ao nível do sistema da fosforilação oxidativa (OXPHOS), originando consequentemente uma deficiente produção de energia. O correto funcionamento do OXPHOS resulta de uma interação coordenada entre o genoma nuclear e mitocondrial. Assim, as doenças mitocondriais podem ser causadas por defeitos moleculares no genoma mitocondrial, no nuclear, ou em ambos, originando doenças da comunicação intergenómica. Estas doenças resultam da perda ou instabilidade do DNA mitocondrial (mtDNA), e podem ser devidas quer a deleções múltiplas, quer a depleção do genoma mitocondrial. As doenças da comunicação intergenómica adquiriram bastante relevância nos últimos anos tendo sido descritos um vasto número de genes associados a estas patologias. Estas doenças podem ser divididas em dois grandes grupos: i) o síndrome das deleções múltiplas do mtDNA, que se caracteriza pelo aparecimento de miopatias oculares e dos membros e ii) o síndrome da depleção do mtDNA, que constitui um grupo de doenças autossómicas recessivas que se manifestam durante a infância ou início da adolescência com três apresentações clínicas distintas, nomeadamente miopática, encefalomiopática e hepatocerebral.

Neste trabalho foram selecionados para estudo molecular 47 doentes clinicamente suspeitos de doenças da comunicação intergenómica. Em 55% dos casos encontrou-se depleção do mtDNA ou deleções múltiplas, 17 dos quais apresentaram depleção “primária” do mtDNA, oito depleção “secundária” do mtDNA e apenas um doente apresentou deleções múltiplas do mtDNA. O espectro mutacional identificado neste estudo encontra-se distribuído pelos seguintes genes: *MPV17*, *DGUOK*, *SUCLA2*, *C10orf2*, *POLG*, e *TAZ,* nos casos com depleção “primária” do mtDNA; *TTC19* e *RARS2* nos doentes com depleção “secundária” do mtDNA; e pelo gene *TYMP*, num doente com deleções múltiplas do mtDNA. Contudo, alguns dos casos estudados continuam sob investigação molecular, devido ao fato de não terem sido identificadas mutações causais, o que significa que a utilização da sequenciação do exoma, poderá permitir esclarecer a etiologia molecular destes doentes. Para além disso, foram testados alguns agentes terapêuticos *in vitro*, de modo a avaliar a sua eficácia, quer ao nível do aumento da produção de ATP, quer quanto à redução das espécies reativas de oxigénio. Estes compostos com potenciais efeitos terapêuticos poderão ter grande impacto na saúde dos doentes e estabelecer novas estratégias terapêuticas para o tratamento destas patologias.

CHAPTER I

INTRODUCTION

INTRODUCTION

1.1. Mitochondria

Mitochondria, from the Greek mitos (thread-like) and khondros (grain or granule), are bacterium-sized organelles found in all nucleated cells. They are dynamic subcellular organelles present in virtually all eukaryotic cells with numerous functions, but it is fair to say that what sets them apart is their role in energy production. To reiterate an often used term, mitochondria are the 'powerhouses of the cell' because they generate more than 90% of a typical cell's adenosine triphosphate (ATP), which is the 'energy currency' of the cell (Schon *et al.*, 2012). They play an important role in various metabolic and developmental processes such as calcium homeostasis, apoptosis and programmed cell death, just to mention some. Mitochondria produce ATP by means of the mitochondrial respiratory chain (MRC) and oxidative phosphorylation (OXPHOS) system, a series of five enzyme complexes embedded in the inner mitochondrial membrane (IMM).

1.1.1. Organization and function of mitochondria

Mitochondria are large organelles, approximately one micron in size, found in the cytosol of all cells. Mitochondrial metabolism is compartmentalized by a two-membrane system: the outer mitochondrial membrane (OMM) and the IMM that envelop an aqueous compartment, the mitochondrial matrix (Figure 1). The space between the inner and outer membranes is referred to as the intermembrane space and has an ionic composition similar to the cytosol. The integrity of these membrane structures is essential for proper mitochondrial function. In addition, specialized domains within the membrane system provide interaction with the rest of the cell in order to coordinate its energy demands (Rosenberg, 2004).

The OMM is responsible for interfacing with the cytosol and its interactions with cytoskeletal elements are important for movement of mitochondria within a cell. This mobility is essential for the distribution of mitochondria during cell division, differentiation, and possibly for positioning of mitochondria to cellular regions under intense demands for energy. This membrane contains a large number of integral proteins, termed porins, and is also involved in the transport of proteins from the cytosol to the interior of mitochondria. Small molecules less than 15 kD are freely diffusible to the intermembranous space, however, larger molecules only enter through the translocase of the outer membrane (TOM) complex, when a signalling sequence to the mitochondria is present.

The IMM is much more selective than the OMM and fold into the matrix to form cristae. A translocase of the inner membrane (TIM) complex can be found, which facilitate the translocation of proteins into the matrix and also the insertion of proteins into the IMM. Here integral membrane proteins are found such as the five complexes of the MRC including the electron transport chains (complexes I-IV), ATP synthase (complex V), and the the adenine nucleotide translocator (ANT). Electron transport through the MRC generates the electrochemical gradient necessary to produce ATP. Key components of the electrochemical gradient include the membrane potential of IMM and the proton gradient .

The matrix contains the mitochondrial genetic system as well as several enzymes, including those for the tricarboxylic acid (TCA) cycle, oxidation of pyruvate and fatty acids and the machinery used in macromolecular synthesis which is required for mitochondria to replicate and function properly (Rosenberg, 2004).

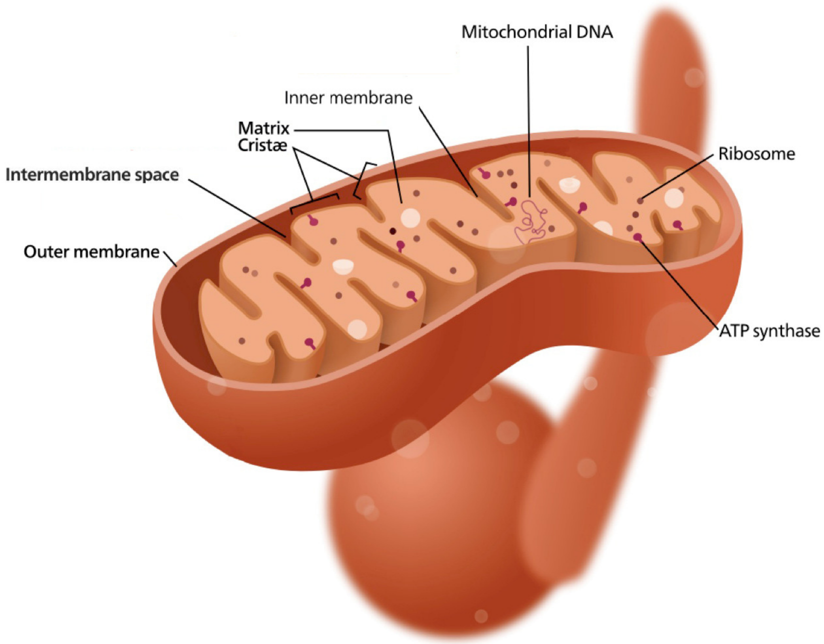


Figure 1 – Schematic representation of mitochondria structure (figure adapted from http://commons.wikimedia.org/wiki/File:Mitochondrion_(standalone_version)-en.svg).

These structural dynamics may serve to regulate mitochondrial metabolism (Dieteren *et al.*, 2011). Mitochondrial internal and external structure varies with cell type and metabolic state and often becomes altered during mitochondrial dysfunction. Mitochondrial morphology also depends on the balance between mitochondrial motility, fission, and fusion. This equilibrium is regulated by dedicated proteins and plays a role in metabolic energy dissipation, turnover of damaged mitochondria, distribution of mitochondria throughout the cell, induction of apoptosis, mitochondrial inheritance during cell division, and defense against aging (Westermann *et al.*, 2010).

The mitochondrial fundamental role is related with the cellular energy metabolism, which includes the TCA, the fatty acid β-oxidation, the urea cycle and the final common pathway for ATP production – OXPHOS (Friedkin and Lehninger 1949; Attardi and Schatz 1988).

Inner mitochondrial membrane lipid milieu

The phospholipid component of the IMM, in which the MRC resides, provides much more than a passive cell scaffold. Alterations of the lipid milieu are increasingly being associated with mitochondrial encephalomyopathies.

Cardiolipin is a dimeric molecule composed of two phosphatidylglycerol moieties connected by a glycerol group (Schlame *et al.*, 2005), is the signature mitochondrial phospholipid and a major component of the IMM, where it is synthesized. Cardiolipin contains four acyl chains, the composition of which depends on the deacylation–reacylation activity of tafazzin, a phospholipid–lysophospholipid transacylase that confers cell-type and tissue specificity (Schlame *et al.*, 2012; Claypool *et al.*, 2012).

The mitochondria-associated membrane (MAM) is a close physical and functional association between the endoplasmic reticulum and mitochondria. These membranes consist of specialized detergent-insoluble lipid raft domains that are rich in cholesterol and sphingolipids, and function as platforms for membrane proteins (Hayashi *et al.*, 2009). MAMs are involved in multiple functions, including lipid transport, cholesterol metabolism, calcium signalling, energy metabolism, apoptosis and mitochondrial dynamics. MAM is a recently described subcellular structure. The structure, although not with the acronym MAM, was first described in 1990 (Vance, 1990).

Mitochondrial transition pore

Most mitochondrial functions involve proteins that control the movement of various molecules and ions across the IMM. The permeability transition defines an increase of IMM permeability to ions and solutes with molecular masses up to 1,500Da, and it is presumed to be mediated by opening of a channel, the permeability transition pore (Bernardi, 2013).

One particularly important ion that must go through this membrane is calcium (Ca^{2+}); once inside the mitochondria, Ca^{2+} regulate cell survival and generation of ATP. Although several Ca^{2+} import mechanisms exist, the best-studied pathway involves a pore-forming protein complex called the mitochondrial calcium uniporter (MCU). This ion channel has an exquisite selectivity, allowing only Ca^{2+} into mitochondria even when other ions outnumber it a million-fold. Mitochondrial Ca^{2+} uptake via the uniporter is crucial to cell metabolism, signaling, and survival (Csordás *et al.*, 2013).

Previously, researchers had identified several genes that are required for the formation of the uniporter, but it had not been established which of those encode the central pore allowing Ca^{2+} to go through. Chaudhuri and collaborators have shown that one of these, a gene called *MCU*, codes for the protein subunit that creates the pore (Chaudhuri *et al.*, 2013). MCU is a transmembrane protein of the IMM with two predicted transmembrane domains connected by a loop that seems to contribute to the selectivity filter (Baughman *et al.*, 2011; De Stefani *et al.*, 2011). MCU likely oligomerizes to form a pore.

1.1.1.1 Mitochondrial dynamics

Mitochondrial network dynamics, much like mitochondrial DNA (mtDNA) replication, is controlled completely by nuclear DNA (nDNA), although likely involves mtDNA–nDNA communication (Youle *et al.*, 2012). Mitochondria form a complex reticulum that is undergoing continual fusion and fission (Figure 2).

Fusion (i) has evolved as a mechanism to promote inter-mitochondrial cooperation, allowing the sharing and dissemination of mtDNA and mitochondrial proteins.

Fission (ii) promotes mitochondrial compartmentalization (Youle *et al.*, 2012), a mechanism that is needed to distribute mitochondria during cell division.

i) Mitochondrial fusion

Mitochondrial fusion is essential to maintain a homogenous organelle population and ensures inter-complementation of mtDNA. Mitochondrial fusion is a two-step process, in which the outer and IMM fuse by separate events. In mammals OMM fusion is controlled by two large membrane GTPase proteins, Mitofusin1 (Mfn1) and Mitofusin2 (Mfn2), whereas IMM fusion is controlled by Optic atrophy 1 (OPA1). It is unknown how the fusion machineries are activated and how mitochondrial content, distribution and division timing are coordinated. The Mitofusins are essential for mitochondrial fusion and their loss of function results in fragmentation of the mitochondrial network (Osellame *et al.*, 2012).

ii) Mitochondrial fission

Mitochondrial division is essential for organelle biogenesis and inheritance and left unregulated can lead to a heterogeneous population of organelles with non-uniform mtDNA distribution, varied ability to produce ATP, increased capacity to generate reactive oxygen species (ROS) and increased susceptibility of cells to undergo apoptosis. Fission is also required for the removal of aged or damaged mitochondria through a specialized form of autophagy, termed mitophagy. While a number of different proteins have recently been proposed to actively contribute to the fission process, Dynamin related protein 1 (Drp1) is almost the only conserved though evolution. Drp1 belongs to the large family of dynamin related GTPases, is dispersed in the cytosol and cycles on and off mitochondria in a guanosine-5'-triphosphate (GTP) dependent manner. It assembles on the OMM in multimeric ring-like structures to facilitate scission of the double membrane. (Osellame *et al.*, 2012).

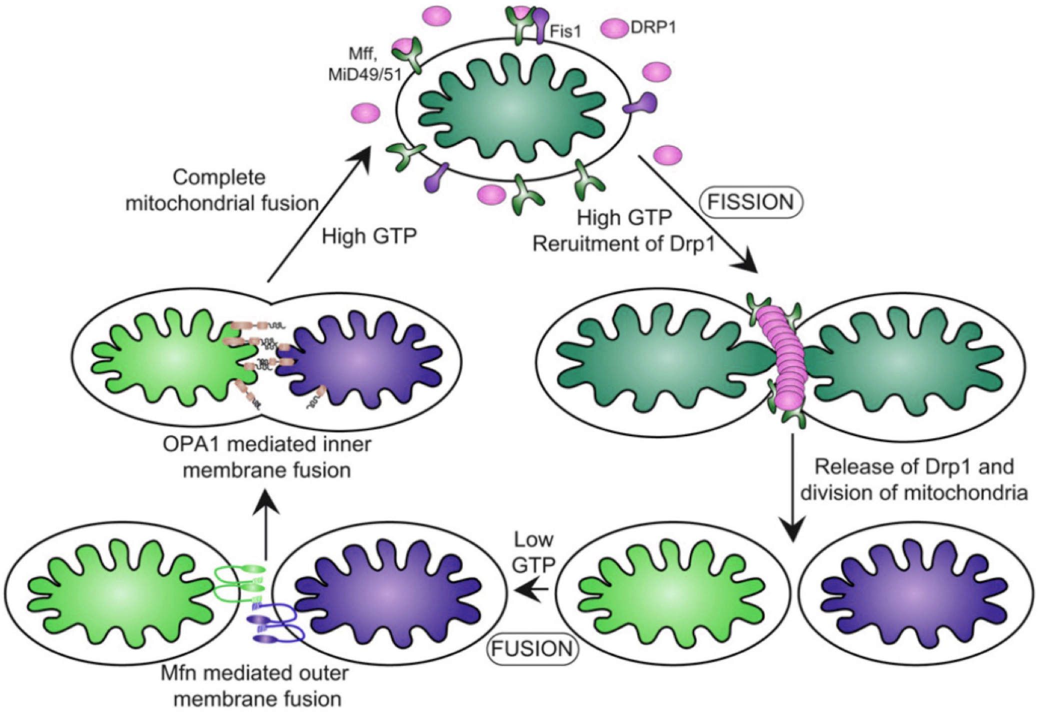


Figure 2 - Mitochondrial dynamics. Steady state mitochondrial morphology requires a balance of fission and fusion events. Organelle division is mediated by Drp1 which forms high molecular weight oligomers on the mitochondrial surface. Once Drp1 is released fission is complete. Mitochondrial fusion is a two-step process that requires outer and inner membrane fusion. Outer membrane fusion is facilitated by mitofusin tethering of adjacent membranes. In high GTP environments, OPA1 isoforms allow inner membrane fusion (figure adapted from Osellame *et al.*, 2012).

1.1.1.2 Mitochondrial biogenesis

Mitochondrial biogenesis is a complex process, driven by a set of nuclear-encoded transcription factors and assisted by transcriptional co-factors, through which the cell equilibrates its energy-harvesting capacity to meet its energetic demands during endurance exercise, caloric restriction, cold exposure, or other cellular stress, such as DNA damage (Andreux *et al.*, 2013).

Considering this transcriptional network, one can distinguish two approaches targeting mitochondrial biogenesis: i) the upstream regulators (for example, sensors), or ii) their downstream effector pathways (for example, transcription factors and co-factors).

i) Energy stress promotes the activation of AMP-activated protein kinase (AMPK) via an increase in the AMP/ATP ratio, whereas energy excess activates the mammalian target of rapamycin (mTOR) pathway either via an increase in levels of amino acids or via the activation of insulin signalling. Rapamycin, 5-aminoimidazole-4-carboxamide riboside (AICAR) and resveratrol simulate energy crisis by inhibiting mTOR or activating AMPK. AMPK-mediated phosphorylation of co-factors (such as peroxisome proliferator-activated receptors-Y (PPARγ) co-activator of proliferator-activated receptor gamma coactivator 1-1α (PGC1α)) and transcription factors (such as forkhead box O (FOXO) proteins) is a preliminary step required for their activation. AMPK also increases the levels of NAD^+, which is a substrate for sirtuins. NAD^+ levels can be increased either by supplying precursors or by inhibiting NAD^+ consuming enzymes (CD38 and poly adenosine diphosphate (ADP) ribose polymerases (PARPs)). The increase in NAD^+ levels activates sirtuin 1 (SIRT1), which subsequently deacetylates PGC1α and FOXO. The acetylation status of PGC1α is counterbalanced by the activity of the histone acetyltransferase GCN5, which is activated via the recruitment of steroid receptor co-activator protein 3 (SRC3). Sirtuin-activating compounds (STACs) can also activate SIRT1 directly (Figure 3A).

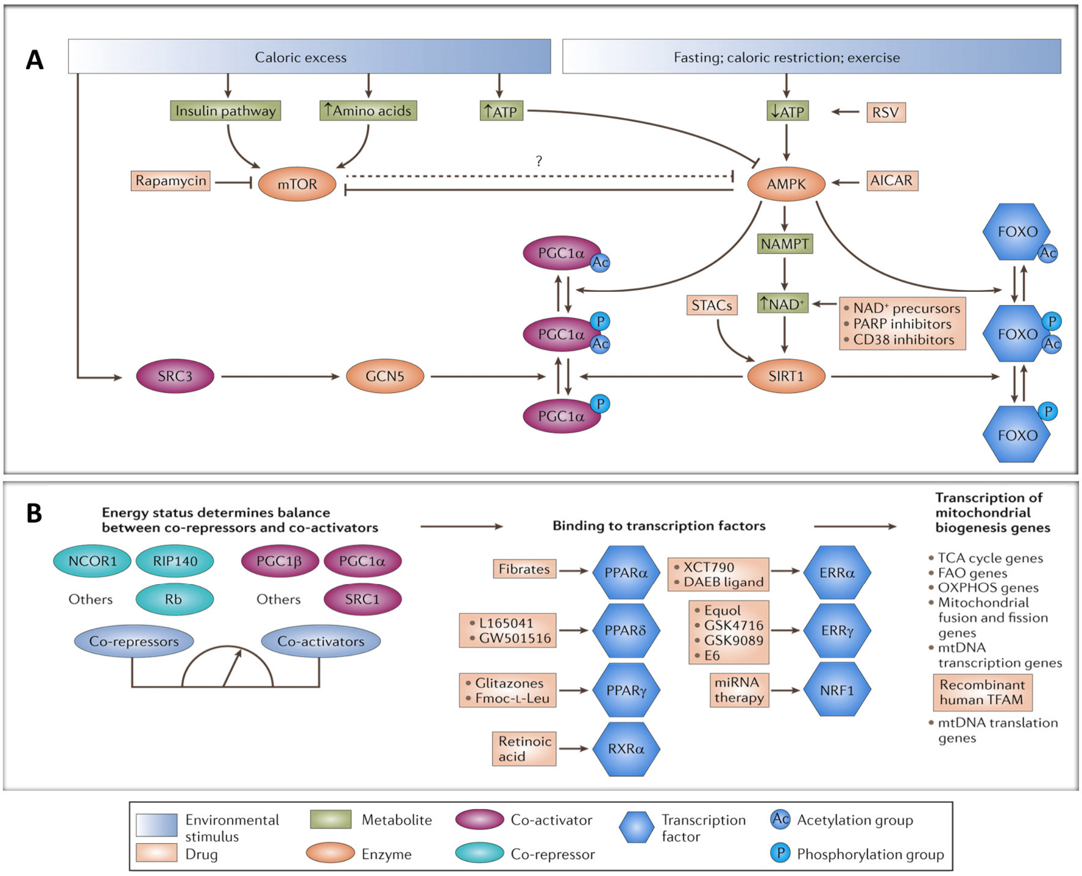


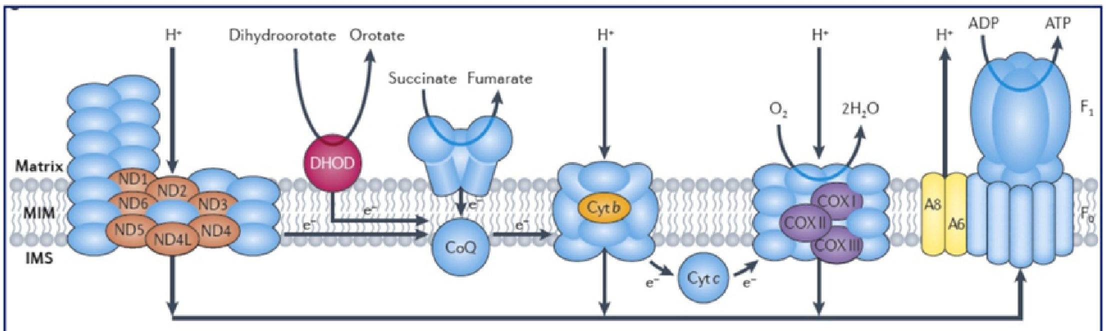
Figure 3 - Pharmacological approaches for targeting mitochondrial biogenesis. A) Upstream sensors of energy status. **B)** Downstream transcriptional factors and co-factors (figure adapted from Andreux *et al.*, 2013). FAO, fatty acid oxidation; NAMPT, nicotinamide phosphoribosyltransferase; NCOR1, nuclear receptor co-repressor 1; OXPHOS, oxidative phosphorylation; RIP140, receptor-interacting protein 140; Rb, retinoblastoma protein; TCA, tricarboxylic acid.

ii) The balance between the activity of co-repressors and co-activators determines the activation of transcription factors involved in mitochondrial biogenesis. Nuclear receptors such as PPARs are ideal drug targets as they are activated upon ligand binding. Likewise, agonist ligands for retinoid X receptor-α (RXRα), the heterodimerization partner of the PPARs, enhance mitochondrial function. Also, several compounds can induce the transcriptional activity of the oestrogen-related receptors (ERRs). The only approach described to modulate the activity of the transcription factor nuclear respiratory factor 1 (NRF1) was a microRNA (miRNA)-based approach. Finally, a recombinant form of human mitochondrial transcription factor A (TFAM) was designed to stimulate the processing of mtDNA (Figure 3B).

1.1.2. Mitochondrial respiratory chain / Oxidative phosphorylation

The OXPHOS system is the main responsible for the production of energy in the cell and is carried out in IMM by the MRC complexes (I, II, III and IV) (Ghezzi *et al.*, 2012). OXPHOS is composed of five enzymatic multiheteromeric, four from the MRC plus complex V, all embedded in the IMM, and two mobile electron shuttles, ubiquinone, a lipoidal quinone, and cytochrome *c*, a heme-containing small polypeptide. The protein subunits of the MRC complexes are assembled together with prosthetic groups and metal-containing reactive centres by a set of chaperones and assembly factors, some of which are specific to each complex. Coenzyme Q (a lipoidal quinone) and cytochrome *c* are also involved in mitochondrial respiration, serving as 'electron shuttles' between the complexes (Wallace, 1999). The OXPHOS system is under the control of two separate genomes, the nuclear and the mitochondrial genomes, and all OXPHOS complexes, except complex II, contain both nDNA and mtDNA encoded polypeptides (Figure 4).

In terms of function, the first two linked events of respiration, i.e. electron transfer and proton pumping, are carried out by the mitochondrial electron transport chain (mETC), a functional supramolecular structure located in the lipid bilayer of the membrane, and composed of four complexes (complex I–IV). In humans, complex I or reduced nicotinamide adenine dinucleotide (NADH) - ubiquinone oxidoreductase, which accomplishes the oxidation of NADH derived by the oxidation of fatty acids, pyruvate and amino acids, contains seven subunits encoded by the mtDNA (subunits *ND1–ND6* and *ND4L*), plus at least 37 nDNA subunits (Smeitink, 2001; Carroll *et al.*, 2003). Among these 44 subunits, 14 are enzymatic 'core subunits' (7 from mtDNA and 7 from nDNA) (Hirst *et al.*, 2011) and the remaing 30 are nDNA accessory subunits thought to maintain complex stability (Angerer *et al.*, 2011). Complex II or succinate-ubiquinone oxidoreductase, which accomplishes the oxidation of flavine adenine dinucleotide ($FADH_2$) derived from fatty acid and the TCA cycle, is composed of only four subunits, all encoded by the nDNA. Complex III or ubiquinol-ferricytochrome c oxidoreductase holds one subunit, cytochrome *b*, encoded by the mtDNA and 10 subunits encoded by the nDNA (Smith *et al*., 2012). Complex IV or cytochrome *c* oxidase (COX) is composed of 14 subunits, three of which are encoded by mtDNA (*COX I–III*) and the other 11 by nDNA. In addition, mETC contains two highly hydrophobic, mobile, small electron carriers, coenzyme Q10 (CoQ10) and cytochrome *c*, both synthesized by nuclear genes. In substance the mETC is especially built to accept electrons from NADH and $FADH_2$, transfer them through a series of oxidation–reduction reactions to molecular oxygen (O_2) to produce water and simultaneously coupling this exergonic reaction to the translocation of protons across the IMM (Saraste, 1999; Di Donato, 2000). Synthesis of ATP from ADP is the second fundamental reaction of the MRC, a process performed by complex V or ATP synthase. ATP synthase is also a genetic mosaic, since it is composed of two mtDNA-encoded subunits (*ATPase 6* and *8*), and at least 17 nDNA-encoded subunits.



Subunits	Complex I	Complex II	Complex III	Complex IV	Complex V
mtDNA-encoded	7	0	1	3	2
nDNA-encoded	37	4	10	11	17
Assembly factors	11	2	4	18	4

Figure 4 – Schematic view of the OXPHOS complexes. Ninety-two structural OXPHOS subunit genes have been identified: 13 encoded by mtDNA and 79 encoded by the nuclear genome. Complex I consists of 44 subunits, seven encoded bt mtDNA and 37 encoded by nDNA. Complex II is encoded entirely by four nDNA subunits. Complex III contains 11 subunits, one encoded by mtDNA and 10 encoded by nDNA. Complex IV consists of three mtDNA-encoded subunits and a further 11 nDNA-encoded subunits. Finally, complex V comprises 19 subunits, 2 encoded by mtDNA and the remaining 17 encoded by nDNA. In addition, nDNA encodes over 39 proteins required for the MRC assembly: 11 nDNA assembly factors of complex I, two of complex II, four of complex III, 18 of complex IV and four of complex V (figure adapted from Shon *et al*., 2012).

As mentioned, the proton electrochemical gradient generated at the mETC level during electron transfer to O_2 creates a polarization of the IMM which is changed back by the proton flux through a proton channel that resides in the F_0 component of ATP synthase. The proton flux drives the condensation of ADP and inorganic phosphate into ATP (Saraste, 1999; Wallace, 1999). Electron transfer across the mETC and ATP synthesis is coupled, or linked. In fact, the MRC works as a proton pump which generates a proton gradient and a membrane potential of about 180mV across the IMM with a negative polarity at the matrix side of the IMM. The proton gradient is utilized by the ATP synthase to phosphorylate matrix ADP. During this process the proton gradient decreases and this activates respiration, i.e. electron transfer (Saraste, 1999).

Hence, the fundamental reaction of life, i.e. O_2 activation and the conservation of energy in cell respiration, is essentially a function of the integrity of the MRC (Babcock and Wilkstrom, 1992). Notably, energy production in mitochondria requires not only a full assembly of functional protein at the level of the IMM, but also a bidirectional flow of information between the nuclear and the mitochondrial genomes to adjust energy production in tissues to different energetic demands (Poyton and McEwan, 1996). Accordingly, many different mutations in mtDNA and nDNA encoding subunits, including nuclear proteins necessary for the proper assembly/stability of the MRC complexes, can produce a wide range of OXPHOS diseases (DiMauro and Schon, 2003; Zeviani and Carelli, 2003; Ghezzi *et al.*, 2012).

1.1.3. The genetic system of mitochondria

The MRC is the only metabolic pathway in the cell that is under the dual control of the mtDNA and nDNA. Therefore, a genetic classification of the mitochondrial diseases distinguishes disorders due to mutations in mtDNA, which are governed by the laxer rules of mitochondrial genetics, and disorders due to mutations in nDNA, which are governed by the stricter rules of Mendelian genetics.

1.1.3.1. Human mitochondrial genome

The human mtDNA is a circular, double-stranded molecule with a striking economy of sequence organization, compressing 37 genes into 16.6 kilobase pairs of DNA (Figure 5). Unsurprisingly, here are no introns and the genes are arranged end to end with little or no intergenic regions. However, there is one sizeable non-coding region (D-loop) that contains a number of important regulatory elements for replication and transcription. MtDNA is devoted to the synthesis of 13 subunits of respiratory complexes I, III, IV, and V. In addition mtDNA encodes the 22 transfer RNAs (tRNAs) and two ribosomal RNAs (rRNAs) necessary for translation within the organelle. The two strands of human mtDNA have different nucleotide compositions and are designated heavy (H) and light (L), according to their buoyant density on cesium chloride gradients. The H-strand contains most of the coding material, including 12 of the 13 protein-coding genes, both rRNAs, and

14 of the 22 tRNAs, whereas the DNA L-strand encodes a single protein and eigh tRNAs (Pearce *et al.*, 2013).

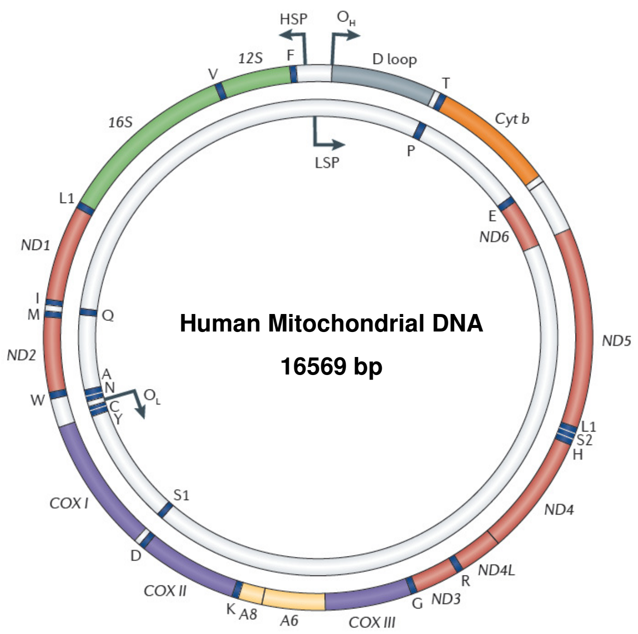


Figure 5 – Mitochondrial DNA. The human mitochondrial genome. The 37 mtDNA - encoded genes include seven subunits of complex I (*ND*) *1*, *2*, *3*, *4*, 4*L*, *5* and *6*, one subunit of complex III (Cyt *b*), three subunits of complex IV (*COX*) *I*, *II* and *III*, two subunits of complex V (*A6* and *A8*), two rRNAs (*12S* and *16S*) and 22 tRNAs (one-letter code). Also shown are the origins of replication of the heavy strand (O_H) and the light strand (O_L), and the promoters of transcription of the heavy strand (HSP) and light strand (LSP) (figure adapted from Schon *et al.*, 2012).

The fact that mtDNA is a compartmentalized extrachromosomal element contributes to its unique genetic features (Figure 6), such as:

i) Heteroplasmy and threshold effects. In contrast to nuclear genes, each consisting of one maternal and one paternal allele, there are hundreds or thousands of mtDNA molecules in each cell (polyploidy) and at cell division the mtDNA is distributed randomly between the daughter cells. In normal tissues, all mtDNA molecules are essentially identical (homoplasmy). Deleterious mutations of mtDNA usually affect some but not all mtDNA (heteroplasmy), and disease expression is determined largely by the proportion of normal and mutant mitochondrial genomes. A critical proportion of mutated mtDNA is necessary before biochemical defects and tissue dysfunction become apparent. This threshold level varies for each mutation and differs between tissues, being lower in tissues highly dependent on OXPHOS metabolism.

ii) Mitotic segregation. During mitosis, mitochondria appear to be randomly segregated. In heteroplasmic cells, the proportion of mutant mtDNA in the daughter cells can therefore change via random genetic drift, and the phenotype may change accordingly. This phenomenon, called mitotic segregation, explains how the clinical phenotype in patients with mtDNA-related disorders may change with age and why, in rapidly dividing cells, particular variants of mtDNA may be gained or lost. The situation is further complicated by counter-selection against deleterious mutant mtDNAs, based on mitochondrial function, and conversely the replicative advantage of some mutant mtDNAs (Holt, 2010).

iii) Maternal inheritance. At fertilization, all mitochondria (and all mtDNA) in the zygote derive from the oocyte (Giles *et al.*, 1980). Therefore mothers carrying an mtDNA point mutation may transmit it to all their children (males and females), but only her daughters will transmit it to subsequent generations. The occurrence of paternal inheritance of mtDNA in humans is an extremely rare event. A single case of paternal transmission of a pathogenic mtDNA mutation have been described in the literature (Schwartz *et al.*, 2002) and has not been noted in any other cases despite systematic investigation (Filosto *et al.*, 2003; Taylor *et al.*, 2003).

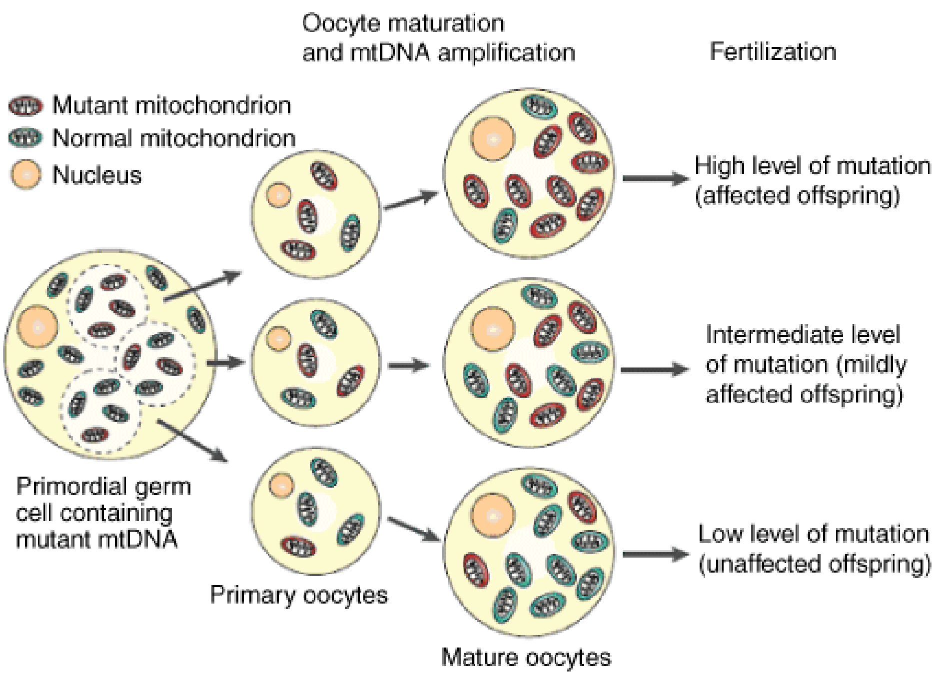


Figure 6 – Mitochondria genetics (figure adapted from http:// acessmedicine.com).

1.1.3.2. Nuclear genome

The mitochondrial proteome is estimated to contain about 1,500 proteins (Calvo *et al.*, 2006). Mitochondria are dependent upon the nuclear genome for the majority of the OXPHOS system and also for maintaining and replicating mtDNA as well as organelle network proliferation and destruction (Figure 7).

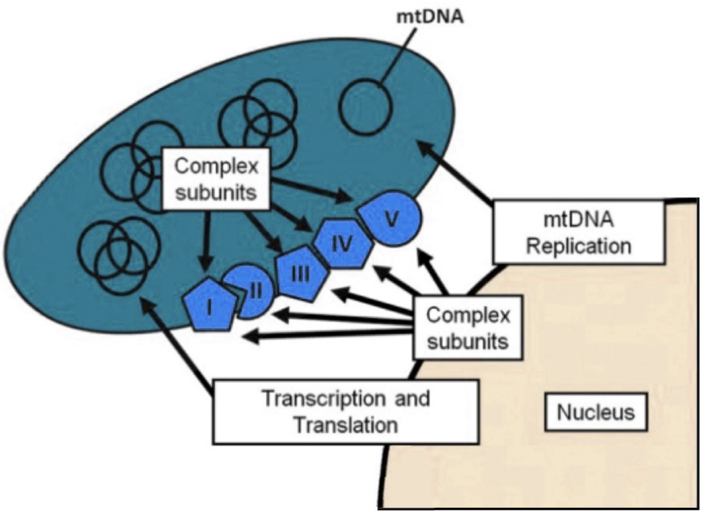


Figure 7 – Interaction between nDNA and mtDNA (figure adapted from Chinnery and Hudson, 2013).

i) mtDNA maintenance

Mitochondria in eukaryotic cells have lost much of their original independent function, with the nucleus performing the role of mtDNA maintenance, including replication and integrity. When the “dialogue” between the two genomes becomes incomprehensible, the resulting diseases are characterized by multiple mtDNA deletions and mtDNA depletion.
Impairment of mtDNA maintenance can be attributable to defects in the replication machinery or in the intramitochondrial pool of deoxynucleoside triphosphates (dNTPs), the DNA building blocks (DiMauro *et al.*, 2013).

mtDNA replication

Unlike nDNA, which replicates with each cell division, mtDNA replicates continuously and independently of cell division (El-Hattab and Scaglia 2013).
MtDNA replication and integrity maintenance is handled entirely by the nDNA. In eukaryotes, mtDNA is replicated in a ‘replisome’ (a DNA/protein complex making up the replication machinery) by a trimeric protein complex composed of a catalytic subunit: polymerase gamma (POLG), a 140 kDa DNA polymerase encoded by

POLG and two 55 kDa accessory subunits, encoded by *POLG2* (Copeland *et al.*, 2012). This enzyme complex performs three activities, DNA polymerase activity, 3'-5' exonuclease/proofreading activity and a 5' dRP lyase activity (required for enzymatic DNA repair). In addition, the replisome also includes the mitochondrial single stranded binding protein (encoded by *mtSSB*), which is involved in stabilizing single-stranded regions of mtDNA at replication forks, enhancing POLG activity. Twinkle is a 5'-3' DNA helicase, which unwinds double-stranded mtDNA, facilitating mtDNA synthesis, as well as acting as a mtDNA primase (an enzyme required to prime nucleotide synthesis) (Figure 8A) (Copeland *et al.*, 2012). Finally, the synergy between mitochondrial transcription factor A (encoded by *TFAM*) and mtDNA copy number suggests that TFAM may act as an mtDNA chaperone (a protein that assists the function of another protein) protecting it against oxidative damage.

Mitochondrial Nucleotide Pool

MtDNA replication occurs throughout the whole cell cycle, depending on specific intramitochondrial dNTP pools. Because mtDNA turns over in post mitotic cells, it is clear that mtDNA is able to replicate in the absence of normal cell cycling, but it may not be completely autonomous (Rotig *et al.*, 2009). A constant supply of dNTP is therefore required for mtDNA replication. dNTPs can be synthesized via either the *de novo* pathway, which is cell cycle-regulated, thereby operative only in S-phase cells or the salvage pathway in which dNTPs are produced by utilizing preexisting deoxynucleosides to synthesize DNA precursors.

As mtDNA synthesis is continuous throughout the cell cycle, the salvage pathway becomes essential for mtDNA maintenance. *TK2*, *DGUOK*, *SUCLA2*, *SUCLG1*, *RRM2B*, and *TYMP* genes encode proteins that maintain the mitochondrial dNTP pool mainly through salvage pathways; therefore, mutations in any of these genes result in depleting the mitochondria of DNA building blocks with subsequent mtDNA depletion (El-Hattab and Scaglia 2013).

ii) mtDNA transcription and translation

Transcription of mtDNA is 'prokaryotic-like' and was thought of a two component system involving a protein complex containing the mitochondrial RNA polymerase (*POLRMT*) and two transcription factors (TFB1M and 2M) (McCulloch *et al.*, 2002; McCulloch *et al.*, 2003). However, recent research indicates that TFB1M does not modulate mtDNA transcription in the presence of TFB2M, rather it acts as a dimethyltransferase which stabilizes the small subunit of the mitochondrial ribosome. RNA transcription is regulated by TFAM

(Metodiev *et al*., 2009). Briefly, each strand is transcribed as a polycistronic precursor mRNA molecule (i.e. the mRNA contains all of the genes in one molecule). L-strand transcription is initiated from the light-strand promoter; however, H-strand transcription initiates from two H-strand promoters: HSP1 and HSP2 (Gaspari *et al*., 2004). Transcript elongation is performed by *POLRMT*, enhanced by both 'transcription elongation factor mitochondrial' (*TEFM*) and termination of mature transcripts is carried out by mitochondrial termination factor 1 (*mTERF1*) (Gaspari *et al*., 2004) (Figure 8B).

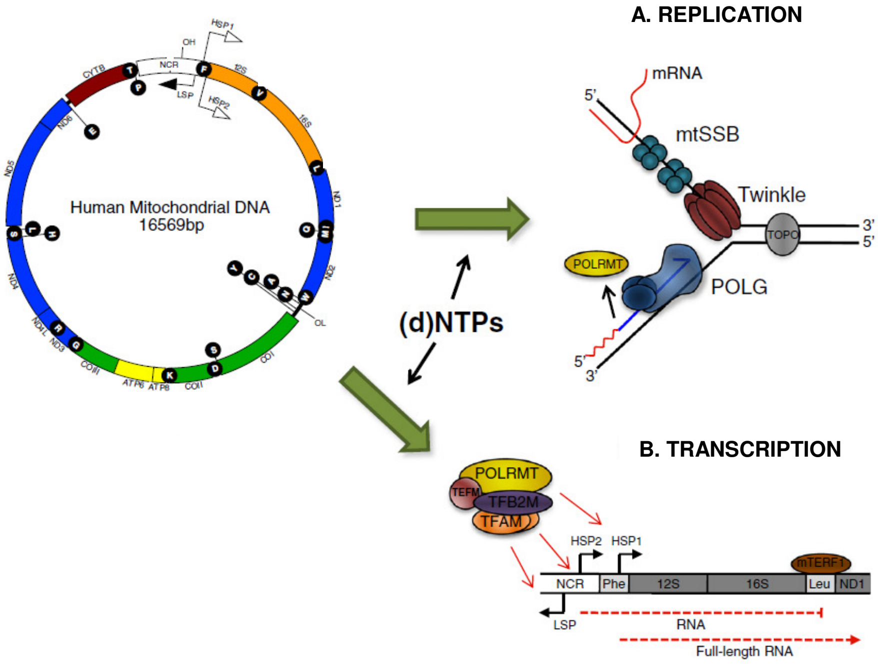


Figure 8 – The human mitochondrial genome and factors involved in mtDNA replication and transcription. A) The core mtDNA replication machinery in mammals includes DNA polymerase POLG, Twinkle helicase, and mtSSB. Replication also involves RNA priming by POLRMT, mtDNA topology alteration by topoisomerases, and possibly RNA incorporation into the lagging strand during leading strand synthesis. **B)** Mammalian mtDNA transcription initiates from three promoters, one for the light strand (LSP) and two for the heavy strand (HSP1, HSP2). The core mtDNA transcription machinery includes RNA polymerase POLRMT and its accessory subunit TEFM, transcription factor TFB2M, and transcription activator TFAM. RNA synthesis beginning from HSP2 is terminated by mTERF1 within the tRNALeu(UUR) gene while transcription started at HSP1 results in near genome-length polycistronic transcripts. The building blocks of nucleic acid are dNTPs (figure adapted from Pearce *et al*., 2013).

Translation of the 13 mtDNA protein coding genes occurs in the mitochondria. The mitoribosomes are partly coded by mtDNA (*MTRNR1* and *MTRNR2*), but require a further 81 nDNA proteins. Translation is initiated by two mitochondrial initiation factors: mtIF1 and mtIF3 (Ma *et al*., 1995; Koc *et al*., 2002). mtIF3 begins initiation by dissociating the 'mitoribosome' (the mitochondrial ribosomes) allowing assembly of the initiation complex (Christian *et al*., 2009). The mRNA is then bound to the small subunit, aligning the start codon to the peptidyl site of the mitoribosome. Peptide elongation is controlled by a number of nuclear-encoded genes, including mitochondrial elongation factor Tu (mtEFTu) (Hammarsund *et al.*, 2001; Ling *et al.*, 1997), which binds the tRNA to the mitoribosome and mitochondrial elongation factor G1 (mtEFG1), required to move the newly added amino acid along one position and allowing amino acid inclusion (Smits *et al.*, 2010). Translation termination is carried out solely by mitochondrial release factor 1a (mtRF1a) (Zhang *et al.*, 1998), which recognizes the stop codons (UAA and UAG) (Soleimanpour-Lichaei *et al.*, 2007) and triggers hydrolysis of the bond between the terminal tRNA and the nascent peptide.

iii) mtDNA arrangement

MtDNA is also packaged in protein–DNA complexes, known as nucleoids (Bogenhagen *et al.*, 2012). Nucleoids of mtDNA are associated with the IMM, spaced evenly along the cristae. In addition to a single mtDNA molecule (Kukat *et al.*, 2011), mtDNA nucleoids contain a number of proteins (Bogenhagen *et al.*, 2012). These nucleoids contain the protein machinery required for DNA replication, transcription, repair and packaging, including the mtDNA polymerase *POLG*, its accessory subunit *POLG2*, the activator of mtDNA transcription (encoded by *TFAM*) as well as mtDNA helicases and binding proteins (*Twinkle* and *mtSSB*, respectively) (Bogenhagen *et al.*, 2012). In addition, mtDNA nucleoids contain chaperone proteins (HSP90-β and HSP70) required for mtDNA stability (Chinnert and Hudson, 2013).

1.2. The genetics of mitochondrial disease

Mitochondrial disorders most often refer to the dysfunction of OXPHOS system leading to deficiency in the ATP production (Ghezzi *et al.*, 2012).. They are a group of genetically and phenotypically heterogeneous disorders with an incidence estimated to be between 1:5,000 and 1:10,000 live births (Schaefer *et al.*, 2004).

The clinical spectrum of mitochondrial disease is diverse (Figure 9), however, tissues where there is a high metabolic demand, such as the central nervous system (CNS), muscle or heart, are typically affected.

Due to the complex interaction between the two cellular genomes, mitochondrial disease can arise through either (i) a primary mtDNA defect (sporadic or maternally inherited) or (ii) a defect in a nuclear-encoded mitochondrial protein (autosomal inheritance) (Chinnery and Hudson, 2013).

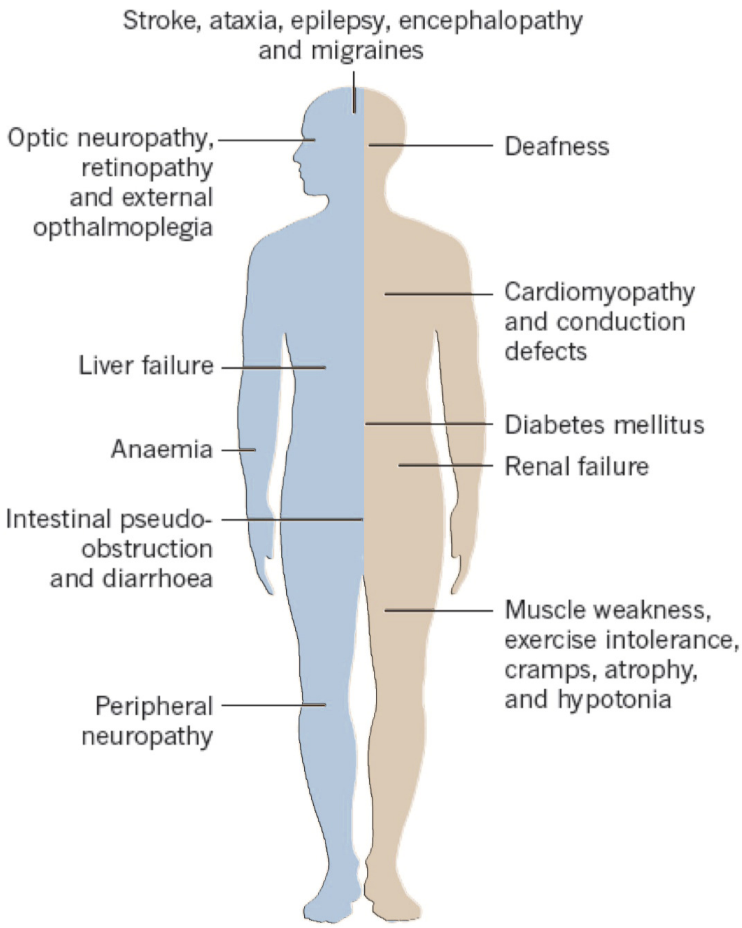


Figure 9 - Clinical spectrum of mitochondrial diseases. Schematic diagram showing the most common clinical manifestations of mitochondrial disorders (figure adapted from Vafai and Mootha, 2012).

1.2.1. Diseases due to mtDNA defects

Since 1988, the birthdate of mitochondrial molecular pathology (Holt *et al.*, 1988; Wallace *et al.*, 1988), the mtDNA circle has become crowded with pathogenic mutations (Figure 10), and the principles of mitochondrial genetics should, therefore, be familiar to the practicing physician.

Diseases due to defects of mtDNA can be divided into two major categories: i) single large-scale rearrangements of mtDNA (e.g. large scale deletions/duplications) and ii) mtDNA point mutations (e.g. tRNA, rRNA or protein-coding genes).

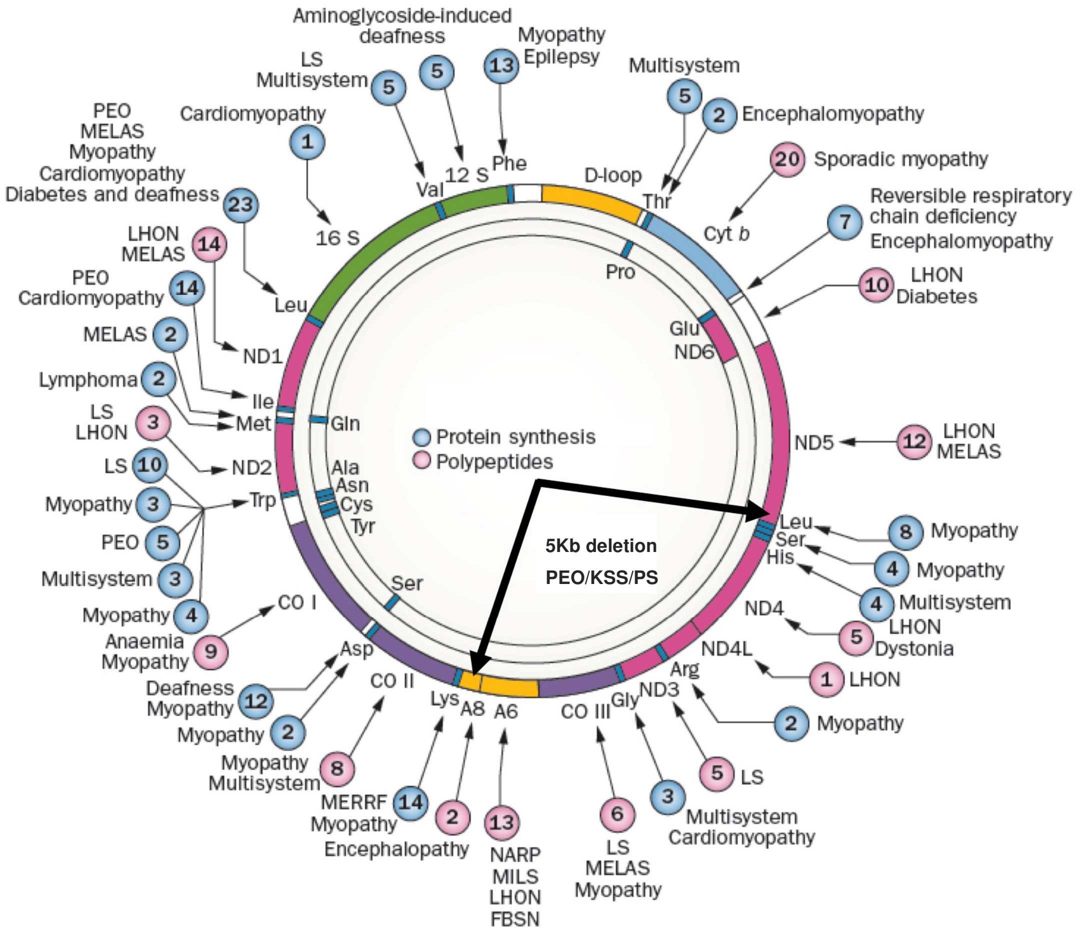


Figure 10 - Morbidity map of the human mitochondrial genome. Schematic map of the 16,569-bp mtDNA, in which coloured sections represent protein-coding genes: seven subunits of complex I (ND; pink sections); one subunit of complex III (cyt *b*; light blue section); three subunits of cytochrome *c* oxidase (CO; purple sections); two subunits of ATP synthase (A6 and A8; yellow sections): 12S and 16S ribosomal RNA (green sections); and 22 transfer RNAs identified by three-letter codes for the corresponding amino acids (blue sections). Diseases due to mutations in genes that impair protein synthesis are indicated as blue circles. Mutated genes that encode respiratory chain proteins are indicated as pink circles. Numbers in circles represent number of mutations reported at the given site. The two black arrows indicate the 5 Kb deletion. Abbreviations: Cyt *b*, cytochrome *b*; FBSN, familial bilateral striatal necrosis; LHON, Leber hereditary optic neuropathy; LS, Leigh syndrome; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonus epilepsy with ragged-red fibres; MILS, maternally inherited Leigh syndrome; NARP, neuropathy, ataxia and retinitis pigmentosa; ND, NADH-dehydrogenase (complex I); PEO, progressive external ophthalmoplegia; KSS, Kearns–Sayre syndrome; PS – Pearson syndrome (figure adapted from DiMauro and Schon 2013).

1.2.1.1. mtDNA rearrangements

Pathogenic rearrangements of mtDNA are typically single large-scale deletions sporadic and not inheritable, and to date over 120 different pathogenic mtDNA deletions have been identified (MITOMAP – http://www.mitomap.org). The ratio of deleted *versus* 'wild-type' molecules is critical to disease etiology, with mtDNA deletions manifesting disease at a lower heteroplasmic threshold (~50–60%) (Rossignol *et al.*, 2003), The exact mechanism of deletion formation is under debate and current research indicates two likely models of deletion formation: replication error and mtDNA repair inefficiency (Schon, *et al.*, 1989; Krishnan *et al.*, 2008). Single deletions of mtDNA have been associated with three sporadic conditions: (i) Pearson syndrome (PS), a rapidly fatal disorder of infancy characterized by sideroblastic anemia and exocrine pancreas dysfunction (Rotig *et al.*, 1990); (ii) Kearns–Sayre syndrome (KSS) (Zeviani *et al.*, 1988), a multisystem disorder with onset before age 20 of impaired eye movements (sporadic progressive external ophthalmoplegia, PEO), pigmentary retinopathy, and heart block; frequent additional signs include ataxia, dementia, endocrine problems (diabetes mellitus, short stature, hypoparathyroidism), lactic acidosis, elevated cerebrospinal fluid (CSF) protein (over 100 mg/dl), and ragged red fibres (RRFs) in the muscle biopsy are typical laboratory abnormalities. (iii) PEO with or without proximal limb weakness, often compatible with a normal life span (Moraes *et al.*, 1989). Deletions vary in size and location, but a "common" deletion of 5 kb is frequently seen in patients and in aged individuals (Figure 10). Duplication of mtDNA can occur in isolation or together with single deletions and have been seen in patients with KSS or with diabetes mellitus and deafness. Duplications and duplications/deletions (as well as the associated phenotypes) are rare and usually transmitted by maternal inheritance (DiMauro *et al.*, 2004).

1.2.1.2. mtDNA point mutations

MtDNA point mutations are usually maternally inherited and they may occur within protein, tRNA, or rRNA genes. However, more than half of disease-related point mutations reported are located within mitochondrial tRNA (mt-tRNA) genes. Phenotypically, point mutations in mitochondrial protein-coding genes specifically affect the function of the MRC complex to which the corresponding protein belongs, whereas mt-tRNA mutations may impair overall mitochondrial translation by reducing the availability of functional mt-tRNAs. Point mutations are mostly heteroplasmic and display considerable clinical heterogeneity. For common point mutations, a typical threshold of 80-90% mutant is required to manifest as disease at the cellular level. There is emerging evidence that mutation levels can change over time, increasing in post-mitotic tissues, such as brain and muscle and decreasing in mitotic tissues including blood (Chinnery and Hudson, 2013). However, an increasing number of pathogenic homoplasmic mutations, often affecting just a single tissue and characterised by incomplete penetrance, are being recognised (Tuppen *et al.*, 2010; DiMauro *et al.*, 2013). Given the high mutational rate of the mitochondrial genome and the presence of numerous family or population-specific polymorphisms, the distinction between neutral mtDNA variant and disease-causing mutation can often be difficult. Below some illustrations will be given:

Leber hereditary optic neuropathy (LHON) is a common cause of inherited blindness that typically presents with bilateral, painless, sub-acute visual failure in young adult males. LHON was the first maternally inherited disease to be associated with an mtDNA point mutation (Wallace *et al.*, 1988). Today, clinical diagnosis is usually confirmed by molecular genetic analysis for one of three 'common' mtDNA mutations, that affect genes coding for complex I subunits of the MRC: m.3460G>A, m.11778G>A and m14484T>C (Carelli *et al.*, 1997). Mitochondrial dysfunction causes a specific loss of retinal ganglion cells (Carelli *et al.*, 2009), whilst preserving the remaining retinal layers. The optic nerve also shows characteristic degeneration and an accumulation of mitochondria suggesting an impairment of axoplasmic transport. LHON mutations are typically homoplasmic; however, not all patients harbouring a pathogenic LHON mtDNA mutation develop visual failure. Studies of LHON have identified common mtDNA variants that may modulate LHON expression (Hudson *et al.*, 2005; Achilli *et al.*, 2012); additionally environmental factors, such as cigarette smoke (Kirkman *et al.*, 2009) and oestrogen levels may play a role (Hudson *et al.*, 2007). However, the majority of research has focused on the identification of a nuclear-encoded susceptibility allele (Hudson *et al.*, 2005; Carvalho *et al.*, 1992; Phasukkijwatana *et al.*, 2010).

Non-syndromic and aminoglycoside-induced sensorineuronal hearing loss is associated with m.1555A>G, a homoplasmic point mutation in the *12sRNA* gene (Prezant *et al.*, 1993). The replacement involves a highly conserved region of *12sRNA*, mutating the molecule to more closely resemble its bacterial homologue. In vitro experiments on m.1555A>G mutant cell lines demonstrated that exposure to aminoglycoside would impair growth; however, not all symptomatic individuals have been exposed to aminoglycoside (Prezant *et al.*, 1993). Surprisingly, given that they make up only 5% of mtDNA, the vast majority of pathogenic mtDNA point mutations occur in the tRNA genes (Zifa *et al.*, 2007; McFarland *et al.*, 2004). In addition, pathogenic tRNA mutations are typically heteroplasmic. Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) is typically a childhood, multisystem disorder. Patients commonly manifest with generalized tonic-clonic seizures, recurrent headaches, anorexia with recurrent vomiting and postlingual hearing loss (Pavlakis *et al.*, 1984; Hirano *et al.*, 1992; Manwaring *et al.*, 2007), but can manifest with impaired: motor ability, vision and mental acuity due to the cumulative effect of multiple stroke-like episodes. MELAS is commonly caused by a A>G transition at m.3243 in *MT-TL1* (80% of cases) (Goto *et al.*, 1990), but is also associated with variants in *MT-ND5* (Santorelli *et al.*, 1997). Biochemically, MELAS manifests as defects of complex I and IV activity; however, care must be taken when interpreting the findings as biochemical results can often appear normal. Myoclonus epilepsy with ragged red fibres (MERRF) is a neuromuscular disorder primarily caused by m.8344A>G in *MT-TK* (Shoffner *et al.*, 1990). Clinically, patients with m.8344A>G present with myoclonus, epilepsy, muscle weakness, cerebellar ataxia and dementia, although neurological symptoms can develop with age (Shoffner *et al.*, 1990). Clinical severity is correlated with patient heteroplasmy with high levels of mutant mtDNA often causing, severe complex I or IV deficiency and occasionally a combined complex I and IV deficiency. Much like MELAS, the genotype–phenotype correlation of m.8344A>G can be extended beyond MERRF. The m.8344A>G mutation has been associated with diverse mitochondrial phenotypes such as Leigh's syndrome. The mutation m.7472insC, affecting *MT-TS*, was first identified in a large Italian family presenting with hearing loss, ataxia and myoclonus (Mancuso et al, 2013). This mutation was later found in several unrelated families, all showing a wide clinical spectrum, including isolated hearing loss, ataxia and MERRF. This mutation has been found at increasing frequencies in families presenting with maternally inherited hearing loss (Chinnery and Hudson, 2013).

1.2.2. Diseases due to nDNA mutations

Nuclear–mitochondrial diseases have been described generally as manifesting in the neonatal period or early infancy, although occasional late-onset patients have been identified. These group of diseases can be classified into four distinct groups: i) disorders resulting from mutations in nuclear-encoded components or assembly factors of the OXPHOS system; ii) disorders resulting from mutations affecting mitochondrial translation and protein synthesis; iii) disorders of the IMM lipid milieu; iv) disorders due to defects in genes controlling mitochondrial network dynamics and v) disorders resulting from a reduction in mtDNA stability.

1.2.2.1. Disorders resulting from mutations in nuclear-encoded components or assembly factors of the OXPHOS system

Isolated complex I deficiency is by far the commonest biochemical defect found in mitochondrial disorders; however, it has the most complex etiology and clinical spectrum (Haack *et al.*, 2012). Complex I deficiency is associated with a broad range of clinical phenotypes ranging from lethal neonatal disease to adult onset neurodegenerative disorders (Loeffen *et al.*, 2000; Lebre *et al.*, 2011). A high level of genetic heterogeneity, coupled with weak genotype–phenotype correlations, makes difficult to predict the genetic basis on pure clinical grounds (Haack *et al.*, 2012). This is important because of the different inheritance patterns and different natural histories of the different genetic causes. However, some patterns are starting to emerge. There are at least 46 nuclear-encoded subunits of complex I (compared with 7 mtDNA encoded subunits) and so it is unsurprising that nDNA mutations have been identified in 14 of the structural subunits. Pathogenic mutations in *NDUFS1* (Benit *et al.*, 2001), *NDUFS3* (Haack *et al.*, 2012; Benit *et al.*, 2004), *NDUFS4* (van den Heuvel *et al.*, 1998), *NDUFS7* (Smeitink *et al.*, 1999), *NDUFS8* (Loeffen *et al.*, 1998), *NDUFV1* (Benit *et al.*, 2001; Schuelke *et al.*, 1999;) *NDUFA10* (Hoefs *et al.*, 2011), NDUFB3 (Haack *et al.*, 2012) and *NDUFA2* (Hoefs *et al.*, 2008) typically manifest as Leigh or Leigh-like syndromes (MITOMAP – http://www.mitomap.org; Shoubridge *et al.*, 2001). Conversely, mutations in *NDUFS2* (Loeffen *et al.*, 2001), *NDUFS6* (Kirby *et al.*, 2004), *NDUFV2* (Benit *et al.*, 2003), *NDUFA1*, *NDUFA11* (Berger *et al.*, 2008) and *ACAD9* (Haack *et al.*, 2010) are typically associated with hypertrophic cardiomyopathy and encephalopathy. In addition, mutations in complex I assembly proteins can manifest as disease: Leigh syndrome (*NDUFAF2* and *NDUFAF5*) (Calvo *et al.*, 2010; Gerards *et al.*, 2010), encephalopathy (*NDUFAF4*) (Saada *et al.*, 2008) and cardioencephalomyopathy (*NDUFAF1*) (Dunningr *et al.*, 2007).

Complex II is encoded only by nDNA and is composed of four polypeptide subunits: *SDH-A*, *B*, *C* and *D*. Mutations in *SDHA* are rare, but are associated with Leigh's syndrome. Surprisingly, mutations in *SDHB*, *SDHC* and *SDHD* appear to be a common cause of inherited paragangliomas and phaeochromocytomas (Baysal *et al.*, 2002).

Complex III deficiency typically causes a severe multisystem early onset disorder, which is recessively inherited and rare (Petruzzella *et al.*, 1998; de Lonlay *et al.*, 2001). Identified mutations in *BCS1L*, a complex III assembly protein, presenting with neonatal proximal tubulopathy, hepatic involvement and encephalopathy. Subsequently, a deletion in human ubiquinone–cytochrome *c* reductase binding protein of complex III (*UQCRB*) was identified in a consanguineous family presenting with hypoglycaemia and lactic acidosis (Haut *et al.*, 2003); and a missense mutation was identified in *UQCRC*, a ubiquinone-binding protein, in a large consanguineous Israeli-Bedoiun kindred (Barel *et al.*, 2008).

More recently, a mutation in *TTC19* (a complex III structural subunit gene) was identified in individuals with a progressive neurodegenerative disorder in late infancy (Ghezzi *et al.*, 2011), expanding the phenotype of complex mutations beyond early infant disorders. Mutations in complex IV result in severe, typically fatal, infantile disease and to date mutations in four complex IV structural subunits have been identified. A homozygous mutation in *COX6BI*, identified in brothers from a consanguineous Saudi Arabian family, presented with gait instabilities visual disturbances, progressive neurological deterioration and leukodystrophic brain changes (Massa *et al.*, 2008). Mutations in *COX10*, a homologue of yeast haem A: farnesyltransferase, are associated with Leigh syndrome (Antonicka *et al.*, 2003; Coenen *et al.*, 2004a) and a multisystem disorder (Antonicka *et al.*, 2003). Atypically, mutations in *COX7B* (Indrieri *et al.*, 2012) are associated with facial dysmorphisms and congenital abnormalities (Zvulunov *et al.*, 1998), and a single mutation in the structural subunit gene, *COX4I2*, was identified in adult Arab Muslim patients with exocrine pancreatic insufficiency, dyserythropoietic anaemia and calvarial hyperostosis (Shteyer *et al.*, 2009). In contrast, a number of mutations have been identified in complex IV assembly factors. Complex IV assembly gene disorders include *SURF1* (Surfeit locus protein 1), associated with Leigh Syndrome (Zhu *et al.*, 1998; Tiranti *et al.*, 1999); *C12ORF62* (chromosome 12 open reading frame 62), associated with fatal, neonatal, mitochondrial IV deficiency (Weraarpachai *et al.*, 2012); *COA5* (cytochrome *c* oxidase assembly factor 5), associated with neonatal hypertrophic cardiomyopathy (Huigsloot *et al.*, 2011) and *FASTKD2*, associated with cytochrome *c* oxidase defective encephalomyopathy (Ghezzi *et al.*, 2008).

Mutations in nDNA-encoded complex V subunit genes also appear very rare. A mutation in *ATP5E* (ATP synthase, H^+ transporting, mitochondrial F_1 complex, epsilon subunit) was identified in an Austrian woman with complex V deficiency (Mayr *et al.*, 2010), and a single gene defect has been identified in the complex V assembly factor gene *ATPAF2*, resulting in impaired complex V activity (De Meirleir *et al.*, 2004).

1.2.2.2. Disorders resulting from mutations affecting mitochondrial protein synthesis

Human disorders associated with protein synthesis deficiency can arise either from mutation in the mitochondrial genome itself (maternal inheritance) or from mutation in the nuclear gene products required for the translation of messenger ribonucleic acids (mRNAs) encoded in the mtDNA (Mendelian inheritance).

In the tRNA maturation process, a subset of the nucleotide bases within a nascent tRNA molecule undergoes specific modifications, which are critical for folding and codon recognition. One of the best understood modifications is pseudouridylation, common to both nuclear and mitochondrial tRNAs. It is proposed that pseudouridylation facilitates base-pairing and base-stacking within the tRNA secondary structure, increasing the overall stability and functional conformation of tRNAs (Patton *et al.*, 2005). Pseudouridine synthase I (PUS1) has been implicated in this process in both cellular compartments. Mutations in *PUS1* gene are causative of mitochondrial myopathy and sideroblastic anemia (MLASA) [OMIM #600462], a rare autosomal recessive disorder characterized by defects in OXPHOS and iron metabolism, with symptoms specific to skeletal muscle and bone marrow (Bykhovskaya *et al.*, 2004). The disease is clinically heterogeneous, which has in part been attributed to the dual function that PUS1 has in nuclear and mitochondrial tRNA maturation, and so perturbation of PUS1 function can impact differentially on the two translation systems (Fernandez-Vizarra *et al.*, 2007).

Defects in tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (TRMU), a mitochondria-specific enzyme that is required for the 2-thiolation on the wobble position of the tRNA anticodon, result in reduced steady-state levels of three tRNAs ($tRNA^{Lys}$, $tRNA^{Gln}$ and $tRNA^{Glu}$) and consequently impaired mitochondrial protein synthesis (Jacobs *et al.*, 2005; Zeharia *et al.*, 2009). Recently, mutations in *TRMU*, were detected in patients with acute liver failure in infancy (Zeharia *et al.*, 2009). All patients harboring mutations in this gene showed combined OXPHOS deficiencies, with (near) normal complex II activity, and a clear defect in mitochondrial translation. Interestingly, it has been hypothesized that human *TRMU* may modulate the phenotypic manifestations of deafness-associated mtDNA mutations (Guan *et al.*, 2006). Impaired tRNA modification is also predicted to underlie the hypertrophic cardiomyopathy and lactic acidosis associated with mutations in human *MTO1* [OMIM #614702] (Ghezzi *et al.*, 2012).

Mutations in methionyl-tRNA formyltransferase (*MTFMT*) are another cause of defective mitochondrial translation (Tucker *et al.*, 2011). Tucker and colleagues described two patients presenting with Leigh syndrome and combined OXPHOS deficiency, whose fibroblasts lacked detectable formylate - fMet-$tRNA^{Met}$. Thus, some residual mitochondrial translation must occur in its absence, as there would be no OXPHOS activity without the protein products of mtDNA.

An early essential step of protein translation involves covalently attaching an amino acid to its cognate transfer RNA. This process, often referred to as tRNA charging, is performed by a highly specialized group of enzymes, the aminoacyl-tRNA synthetases (ARSs). Reflecting their fundamental importance, ARSs are ubiquitously expressed enzymes that are found in all domains of life. Thirty-six ARSs are sufficient to aminoacylate all tRNAs in humans: 16 act exclusively in the cytoplasm, 17 act exclusively in the mitochondria, and three are bi-compartmental. Although few mutations have been reported in the cytosolic ARSs, an increasing number of mitochondrial ARS mutations have been linked to highly tissue-specific diseases in both humans and mice.

An intriguing observation is that mutations of genes encoding mitochondrial ARSs are usually associated with specific clinical syndromes. For instance, leukoencephalopathy with brainstem and spinal cord involvement and high lactate (LBSL) is caused by *DARS2* mutations (440 published cases) [OMIM #611105] (Scheper *et al.*, 2007), pathogenic mutations in mitochondrial arginyl tRNA synthetase (*RARS2)* cause pontocerebellar hypoplasia type 6 (PCHD-6) (seven published cases) [OMIM #611523] (Edvardson *et al.*, 2007), and leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL) is associated with *EARS2* mutations (Steenweg *et al.*, 2012). Furthermore, complex rearrangements in *MARS2* were recently shown to cause autosomal recessive spastic ataxia with leukoencephalopathy (ARSAL) in 54 individuals (Bayat *et al.*, 2012). In that report, complex I activity, MARS2 protein levels and mitochondrial protein synthesis were reduced in both patients and Drosophila mutants. However, in patient-derived fibroblasts steady-state levels of the cognate tRNA were similar to controls. Interestingly, the patients and the flies displayed increased production of ROS. The resulting progressive neurodegeneration and myopathy in the flies was partially suppressed by antioxidant treatment, implying a potential therapeutic avenue for ARSAL patients, and possibly other ARSs deficiencies.

Although currently fewer in number, patients with mutations in *YARS2*, *HARS2*, *AARS2*, *SARS2* and *FARS2* also display distinct phenotype–genotype relationships. *YARS2* was identified as the affected gene in two families with myopathy, lactic acidosis, and sideroblastic anemia (MLASA) [OMIM #613561] (Riley *et al.*, 2010). *HARS2* has been found mutated in one family with Perrault syndrome [OMIM #614129], a sex-influenced disorder characterized by sensorineural deafness in both males and females, and ovarian dysgenesis in females (Pierce *et al.*, 2011). Two mutations in *AARS2* have been reported to cause fatal perinatal or infantile hypertrophic cardiomyopathy [OMIM #614096] (Götz *et al.*, 2011). A recessive *SARS2* mutation was associated with a infantile multisystem mitochondrial cytopathy defined by hyperuricemia, pulmonary hypertension, renal failure in infancy, and alkalosis - HUPRA syndrome [OMIM #613845] (Belostotsky *et al.*, 2011). Finally, mutations in *FARS2* have been recently associated with mitochondrial encephalopathy Alpers type, thus expanding the genetic cause of Alpers Huttenlocher syndrome (AHS) (previously only associated with mutations in *POLG* or *TWINKLE*) (Elo *et al.*, 2012; Shamseldin *et al.*, 2012). Despite our increasing awareness of pathological ARS mutations, the mechanisms underpinning the specific clinical–genetic associations are unknown.

Proper assembly of mitochondrial ribosomal protein (MRP) into ribosomes is critical for efficient translation. MRPS16 and MRPS22 are components of the mitoribosome. Mutations in these genes are known to cause severe, infantile, lactic acidosis, developmental defects in the brain, and facial dysmorphisms (*MRPS16*) and fatal neonatal hypertrophic cardiomyopathy and kidney tubulopathy (*MRPS22*) (Saada *et al.*, 2007).

Recently, a mutation in *MRPL3* was identified in a patient with hypertrophic cardiomyopathy and psychomotor retardation. The mutation altered the stability of MRPL3 and resulted in defective assembly of the large ribosomal subunit with a severe decrease of mitochondrial translation [OMIM #614582] (Galmiche *et al.*, 2011).

Mutations in genes encoding components of the mitochondrial translation elongation machinery are yet another established cause of encephalopathy and other organ failures. These include elongation factors EF-Tumt (*TUFM*) (Valente *et al.*, 2007), EF-Tsmt (*TSFM*) (Smeitink *et al.*, 2006), and EFG1 (*GFM1*) (Antonicka *et al.*, 2006; Coenen *et al.*, 2004b; Smits *et al.*, 2011; Valente *et al.*, 2007). Patients typically present in infancy and die in childhood or infancy. They often have profound OXPHOS deficiencies, which are invariably associated with decreased mitochondrial translation. Nevertheless, clinical symptoms vary greatly between patients, even between those with mutations in the same gene. Mutations in EFG1 result in encephalopathy presenting either with or without liver involvement [OMIM #609060] (Antonicka *et al.*, 2006). Remarkably, in one patient mitochondrial protein synthesis was impaired in fibroblasts but not in muscle. The phenotypic differences might result from mutation-specific effects on the stability of the protein in different tissues, or variation in the abundance of other elongation factors (Antonicka *et al.*, 2006).

Indeed, two studies have shown that the relative abundance of elongation factors is important in determining mitochondrial translation efficiency (Antonicka *et al.*, 2006; Smeitink *et al.*, 2006). A fatal mutation in *TUFM* was identified in a patient who presented with lactic acidosis, a diffuse cystic leukoencephalopathy, and polymicrogyria [OMIM #610678] (Valente *et al.*, 2007). The mutation, located in the tRNA binding region of EF-Tumt, impairs the formation of a ternary complex with GTP and aminoacyl-tRNA, leading to a severe defect in protein synthesis (Valente *et al.*, 2009). Three unrelated pediatric cases share the same mutation in *TSFM*, encoding EF-Tsmt, yet the clinical features were quite different, being associated with either mitochondrial encephalomyopathy or hypertrophic cardiomyopathy, or a combination of both [OMIM #610505] (Smeitink *et al.*, 2006).

Two mammalian proteins have been proposed to act as translation activators. Translational activator of *COX I* (TACO1) is necessary for the efficient translation of *COXI*, and mutations in the corresponding gene are associated with late-onset Leigh syndrome and COX deficiency [OMIM #612958] (Weraarpachai *et al.*, 2009). The second potential translation activator is leucine-rich pentatricopeptide repeat containing protein (LRPPRC), which is a loose homolog of Pet309, the yeast mitochondrial translation activator of *COXI* and *COXIII*. Mutations in *LRPPRC* have been identified as the cause of French–Canadian variant of Leigh syndrome (LSFC) [OMIM #220111] (Mootha *et al.*, 2003; Xu *et al.*, 2004). A recent study outlines the effects of a cardiac muscle-specific knockout mouse model of LRPPRC (Ruzzenente *et al.*, 2012). Results indicated that the lack of LRPPRC activity leads to reduced steady-state levels of most mitochondrial mRNAs and suggested that LRPPRC functions to maintain the stability of pools of untranslated mRNAs.

1.2.2.3. Disorders of the inner mitochondrial membrane lipid milieu

Cardiolipin deficiency was first documented in the early 2000s in cultured fibroblasts from patients with Barth syndrome (BTHS) is an X-linked mitochondrial myopathy and cardiopathy, with neutropenia and growth retardation (Vreken *et al.*, 2000; Schlame *et al.*, 2003). The tafazzin (*TAZ*) gene responsible for this disorder encodes a family of proteins (“tafazzins”) that are homologous to phospholipid acyltransferases (Bione *et al.*, 1996). Analysis of phospholipids in target tissues from patients with BTHS and *TAZ* mutations, patients with BTHS-like syndromes without *TAZ* mutations, and both normal and disease controls showed decreased cardiolipin in all tissues from genetically proven BTHS patients only (Schlame *et al.*, 2002). Cardiolipin was affected selectively and other phospholipids were normal, and the molecular composition of cardiolipin was altered in all tissues from BTHS patients.

Since then, four new disease entities characterized by deficiencies in different phospholipids have been identified in rapid succession. These entities are: Sengers syndrome, Megaconial encephalomyopathy, 3-methylglutaconic aciduria, deafness, and Leigh syndrome-like encephalopathy (MEGDEL) and childhood myoglobinuria, which are due to mutations in *AGK*, *CHKB*, *SERAC1* and *LPIN1* genes, repectively.

Similar to BTHS, Sengers syndrome primarily affects heart and muscle, with the distinctive additional clinical feature of congenital cataracts, presenting mutations in the acylglycerol kinase (*AGK*) gene (Mayr *et al.*, 2012). Acylglycerol kinase catalyses the phosphorylation of diacylglycerol and monoacylglycerol to form phosphatidic acid or lysophosphatidic acid. Notably, as phosphatidic acid is a precursor of cardiolipin, it thereby provides a point of convergence in Sengers syndrome and BTHS, which could explain some of the clinical similarities between the two disorders.

In 1998, a new condition characterized by congenital myopathy and mental retardation was reported (Nishino *et al.*, 1998). The unusual muscle morphology, which included giant mitochondria that were displaced to the periphery of the fibres, was later observed in spontaneously mutant dystrophic mice that harboured changes in the gene encoding choline kinase-β (*CHKB*) (Nishino *et al.*, 1998). Sequencing of *CHKB* in 15 patients with the disorder revealed 11 pathogenic mutations (Mitsuhashi *et al.*, 2011). CHKB catalyses the first step in the biosynthesis of phosphatidylcholine, a phospholipid that is formed through a biosynthetic pathway within the MAM. The relationship between phospholipid abnormality and mitochondrial dysfunction could, therefore, be mediated through the MAM. Several proteins involved in mitochondrial dynamics are also integral parts of the MAM (Schon *et al.*, 2012), raising the possibility that MAM dysfunction might explain both the increased size and the intracellular displacement of mitochondria in patients with *CHKB* mutations (Gutierrez-Rios *et al.*, 2012).

The role of the MAM in the etiology of mitochondrial diseases that are caused by altered IMM phospholipid composition was confirmed in another disorder, termed MEGDEL (Wortmann *et al.*, 2009; Wortmann et al., 2010). In this case, whole-exome sequencing revealed mutations in *SERAC1*. The SERAC1 protein is localized in the contact sites between the endoplasmic reticulum and mitochondria and catalyzes the remodelling of phosphatidylglycerol (Wortmann *et al.*, 2012). Analysis of fibroblasts from patients with MEGDEL revealed alteration in the distribution of phosphatidylglycerol species and in the composition of cardiolipin subspecies. Quantitative or qualitative alterations in cardiolipin could, therefore, be a common denominator in the pathogenesis of disorders other than BTHS (DiMauro *et al.*, 2013).

1.2.2.4. Disorders due to defects in genes controlling mitochondrial network dynamics

Mutations in *OPA1* are primarily a cause of optic atrophy (Carelli *et al.*, 2009), but additional phenotypes, such as deafness and neuromuscular disease, have also been seen. Interestingly, mutations in *OPA1* also appear to cause the formation of mtDNA deletions, indicating that *OPA1* is also important to mtDNA maintenance. Much like *OPA1*, defects in *MFN2* cause a disturbance of mtDNA maintenance through impairment of mitochondrial network dynamics (Carelli *et al.*, 2009). Mutations in *MFN2* are typically associated with Charcot-Marie-Tooth disease (CMT2A) and hereditary motor and sensory neuropathy (Carelli *et al.*, 2009). *DNM1L* (dynamin 1-like), another GTPase, is required for fission of mitochondria (Smirnova *et al.*, 1998). To date, only a single *DNM1L* mutation has been identified in an infant presenting with both defective mitochondrial and peroxisomal fission (Waterham *et al.*, 2007). The patient presented in the first days of life with severe microcephaly, abnormal brain development, optic atrophy with hyperplasia and lactic academia (Waterham *et al.*, 2007).

1.2.2.5. Disorders resulting from a reduction in mtDNA stability

The nuclear-mitochondrial intergenomic communication is crucial for the cellular regulation of mtDNA integrity and copy number and correct mitochondrial protein production. As all the factors responsible for mtDNA replication and repair are encoded in the nucleus, it is not surprising that mutations in nuclear genes can disrupt mtDNA integrity causing qualitative (multiple deletions) or quantitative (depletion) molecular abnormalities. The “mitonuclear” cross-talk has gained increased relevance in the past years and since then many genes have been identified as being involved in this group of mitochondrial diseases. This topic will be further detailed in section 1.3.

1.3. Intergenomic-communication disorders

The cross-talk between the two genomes is crucial for the cellular regulation of mtDNA integrity and copy number and correct mitochondrial protein production therefore mutations in genes involved in mitochondrial replication and maintenance can disrupt the integrity of the mitochondrial genome, causing intergenomic-communication disorders. Multiple deletions, depletion of mtDNA or a combination of both phenomena (qualitative/quantitative lesions) in critical tissues, are the hallmarks of these disorders.

1.3.1 Clinical manifestations and molecular etiologies of disorders affecting mtDNA integrity

Maintenance of mtDNA is controlled by an intricate homeostatic network, whose effectors are the various components of the mitochondrial replicosome and the many enzymes and carrier proteins that provide the mitochondrion with a balance supply of deoxyribonucleotides (Figure 11).

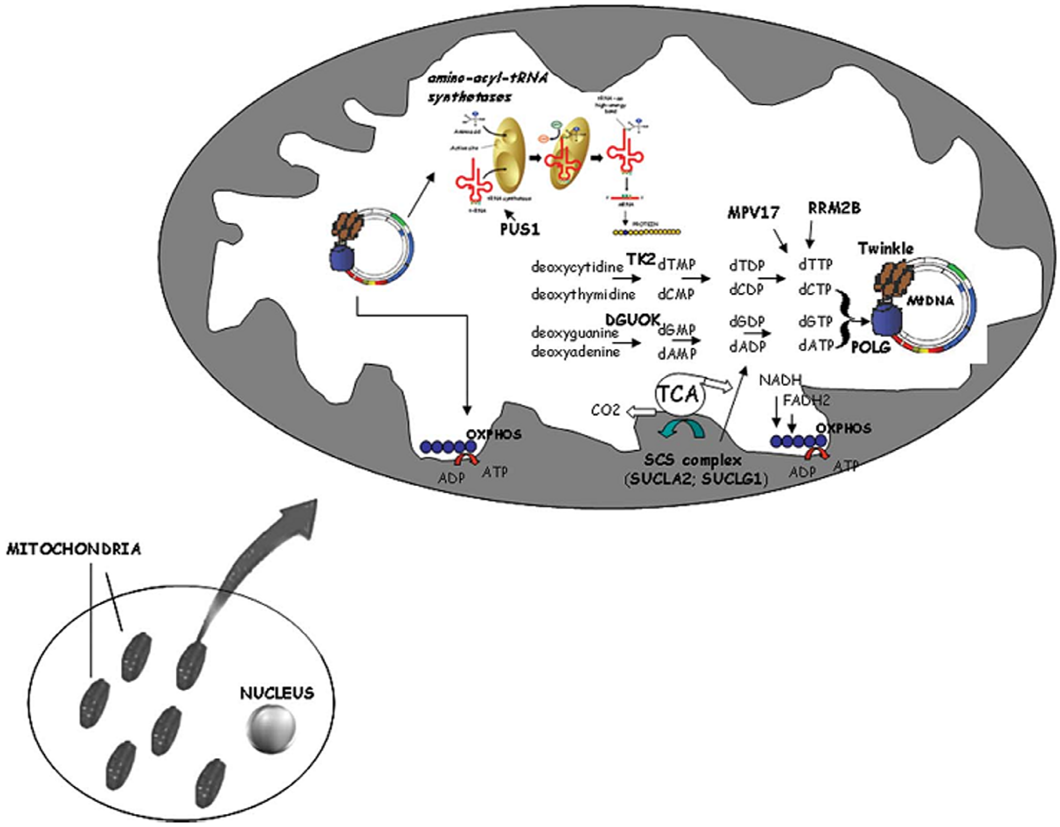


Figure 11 - Schematic overview of the mitochondrion and the mitochondrial disease genes affecting mtDNA integrity. Zooming on the mitochondrion allows to appreciate genes (namely, *POLG1* and *TWINKLE*) thought to be involved in replication of mtDNA, those assumed to affect the metabolism of the mitochondrial dNTP pool (via progressive phosphorylations of dNTPs), and those belonging to the tricarboxylic acid cycle (TCA) and affecting the OXPHOS complexes. Moreover, the supposed role of genes involved in the complex machinery of mitochondrial protein synthesis (including the aminoacyl-tRNA synthetases).

As all of the factors are nDNA encoded, it is not surprising that mutations in genes involved in mitochondrial replication and maintenance can disrupt the integrity of the ‘‘tiny’’ mitochondrial genome (Hirano *et al.*, 2001) leading to multiple deletions or depletion (Spinazzola *et al.*, 2005). The mitonuclear cross-talk has gained increased relevance in the past years and since then many genes have been identified as being involved in these diseases.

In the following section we will briefly review the main clinical manifestations and molecular etiologies associated with mtDNA multiple deletions and mtDNA depletion syndromes. An overview of the main phenotypes and associated mutations is illustrated in Table 1.

Table 1 - Major phenotypes of the disorders affecting mtDNA integrity and the associated genes with the number of described mutations (source HGMD Professional, Database www.hgmd.cf.ac.uk/).

Major Phenotypes	Genes	Number of Mutations	
Progressive External Ophthalmoplegia – PEO	*POLG*	65	**Mitochondrial DNA Multiple Deletions Syndrome**
	POLG2	1	
	C10orf2	39	
	SLC25A4	5	
	*MGME1**	2	
	*TK2**	2	
	RRM2B	11	
	DGUOK	2	
Sensory Ataxic Neuropathy, Dysarthria and Ophthalmoparesis – SANDO	*POLG*	14	
	SLC25A4	1	
SpinoCerebellar Ataxia – Epilepsy syndrome – SCAE	*POLG*	1	
	C10orf2	4	
Mitochondrial NeuroGastroIntestinal Encephalomyopathy – MNGIE	*Tymp**	81	**Mitochondrial DNA Depletion Syndrome**
	RRM2B	2	
	*POLG**	1	
Hepatocerebral – Mitochondrial Depletion Syndrome	*POLG*	8	
	C10orf2	3	
	DGUOK	51	
	MPV17	28	
	TK2	1	
Myopathic – Mitochondrial Depletion Syndrome	*TK2*	34	
	POLG	1	
	RRM2B	3	
	DGUOK	1	
Encephalomyopathic – Mitochondrial Depletion Syndrome	*RRM2B*	14	
	SUCLA2	8	
	SUCLG1	13	
	TK2	1	
	C10orf2	1	
Alpers-Huttenlocher Syndrome – AHS	*POLG*	54	

*Presence of multiple deletions and mtDNA depletion.

1.3.1.1. Mitochondrial DNA multiple deletion syndrome

Mitochondrial diseases associated with the presence of multiple deletions of mtDNA are mostly autosomal dominant, occurring most often in adulthood. The size and terminals deletions are variable from one individual to another within the same family.

The main clinical manifestations associated with multiple deletions will be described along with molecular etiologies.

PEO (Autosomal Dominant or Recessive Progressive External Ophthalmoplegia)

The most common clinical features include adult-onset of weakness of the external eye muscles, bilateral ptosis, proximal muscle weakness wasting and exercise intolerance. Additional symptoms are variable, and may include cataracts, hearing loss, sensory axonal neuropathy, ataxia, depression, hypogonadism, and Parkinsonism. Less common features include mitral valve prolapse, cardiomyopathy, and gastrointestinal dysmotility. Both autosomal dominant and autosomal recessive inheritance can occur; autosomal recessive inheritance is usually more severe (Filosto *et al.*, 2003; Luoma *et al.*, 2004). The multiple deletions associated with PEO are exclusively found in muscle tissues of patients. Mutations in the *POLG*, *POLG2*, *C10orf2*, *SLC25A4*, *MGME1*, *TK2, RRM2B* and *DGUOK* genes have been linked with PEO. Below a small description of the most important genes involved in the molecular etiologies of PEO can be found.

i) POLG

Human mitochondria contain a single DNA polymerase, POLG, nuclear encoded and solely responsible for mtDNA replication and repair in mitochondria. POLG is composed of a catalytic subunit, POLGA, which possesses both polymerase and proofreading exonuclease activities and an accessory subunit, POLGB, which increases enzyme processivity (Lee *et al.*, 2009). The POLG holoenzyme functions in conjunction with the mtDNA helicase and the mitochondrial single-stranded DNA- binding protein to form the minimal replication apparatus (Korhonen *et al.*, 2004). It was generally accepted that mutations within the mtDNA were the major cause of mitochondrial diseases; however this view is changing as several of these have been linked to ineffective mtDNA replication by POLG Mutations affecting the catalytic subunit POLGA, encoded by the nuclear gene *POLG* are a major cause of mitochondrial disease, being highly heterogeneous: PEO, Parkinsonism, AHS, MNGIE, SANDO and SCAE, and usually is associated with multiple mtDNA deletions (Horvath *et al.*, 2006). *POLG* mutations have been shown to be associated with all types of inheritance. The unique features of mitochondrial physiology are in part responsible for this variability but POLG structure and function add to the riddle of how one gene product can demonstrate autosomal recessive and autosomal dominant transmission. POLGA is a key player in mtDNA maintenance that is absolutely necessary for mtDNA replication from an early stage in embryogenesis (Hance *et al.*, 2005).

In autosomal dominant PEO (adPEO) due to *POLG* mutations (most frequent), prominent features are severe dysphagia and dysphonia, and, occasionally, a movement disorder including Parkinsonism, cerebellar dysfunction, and chorea. Recessive mutations of *POLG* are responsible for sporadic and autosomal recessive PEO (arPEO), as well as the syndromes referred above. Mutations in this gene can be also associated to the hepatocerebral form of MDS, namely AHS (Horvath *et al.*, 2006).

The *POLG* gene is located at chromosome 15, comprises 23 exons spanning 18.55 Kb. The gene was identified in 1996 (Ropp *et al.*, 1996) but only in 2001 the first pathogenic mutation was described. Since then more than 150 mutations have been reported and *POLG* gene is considered a hot-spot for mutations in mitochondrial diseases (Chinnery *et al.*, 2008). So far, 65 mutations have been described as being associated with PEO.

ii) POLG2

MtDNA is replicated by POLG, which is composed of a 140-kD catalytic subunit (encoded by *POLG*) and a 55-kD accessory subunit (*POLG2*). The accessory subunit increases enzyme processivity therefore it is not surprising that failure in this processivity leads to the accumulation of mtDNA deletions.

The *POLG2* gene is located at chromosome 17, comprises 8 exons spanning 19.28 Kb. In 2006 the first pathogenic mutation was described as being a cause of adPEO (Longley *et al.*, 2006). Since then, 12 mutations in *POLG2* have been reported associated with unspecific mitochondrial phenotype.

iii) C10orf2 (TWINKLE)

The mitochondrial helicase/primase encoded by *C10orf2* gene is also responsible for the adPEO (Spelbrink *et al.*, 2001). Mutations in *C10orf2* may be of variable severity, being associated with clinical presentations ranging from late-onset ‘‘pure’’ PEO, to PEO complicated by proximal limb and facial muscle weakness, dysphagia and dysphonia, mild ataxia, and peripheral neuropathy. Recessive *C10orf2* mutations were also described in patients with hepatocerebral form of depletion (Hakonen *et al.*, 2007).

The *C10orf2* gene is located at chromosome 10; it comprises 5 exons spanning 6.38 kb. The first pathogenic mutation was reported in 2001 (Spelbrink *et al.*, 2001) to be associated with PEO and since then 39 pathogenic mutations have been reported to be associated with this phenotype.

iv) SLC25A4

This gene, coding for the muscle-heart-specific mitochondrial ANT, is a member of the mitochondrial carrier subfamily of solute carrier protein genes (Kaukonen *et al.*, 2000). ANT is the most abundant mitochondrial protein and in its functional state, it is a homodimer of 30-kD subunits embedded asymmetrically in the IMM. The dimer forms a gated pore through which ADP is moved from the matrix into the cytoplasm. There are three recognized isoforms of this protein.

Mutations in this gene have been shown to be responsible for the adPEO and have been also associated with a relatively mild, slow progressive myopathy, with little or no extra-muscular symptoms.

The *SLC25A4* gene was identified in 2000 (Kaukonen *et al.*, 2000), it is located at chromosome 4, comprises 4 exons spanning 4.04 Kb. The first pathogenic mutations were described in 2000 and since then only eight mutations have been reported (five of them associated with PEO).

v) MGME1 (C20orf72)

The mitochondrial genome maintenance exonuclease 1 gene has recently been described (Kornblum *et al.*, 2013). It is located at chromosome 20, comprises 4 exons and encodes a mitochondrial RecB-type exonuclease belonging to the PD–(D/E)XK nuclease superfamily and it is essential for effective mtDNA synthesis and may have a redundant role in mtDNA repair. It is the first identified mitochondrial exonuclease shown to be involved in mtDNA replication, likely through the processing of displaced DNA or RNA-DNA flap-like structures during mtDNA synthesis (Kornblum *et al.*, 2013).

Only two mutations affecting this mitochondrial exonuclease have been described in a single report in three unrelated families with a mitochondrial syndrome characterized by PEO, emaciation and respiratory failure. These mutations affect proper mtDNA maintenance and are a clinically relevant cause of mtDNA disorders. Muscle biopsies of the identified patients showed mtDNA depletion and multiple mtDNA deletions.

SANDO (Sensory Ataxic Neuropathy, Dysarthria and Ophthalmoparesis)

SANDO is an autosomal recessive systemic disorder characterized mainly by adult onset of sensory ataxic neuropathy, dysarthria, and ophthalmoparesis. The phenotype varies widely, even within the same family, and can include myopathy, seizures, and hearing loss, but the common clinical feature appears to be sensory ataxia (Milone and Massie, 2010). Mutations in the *POLG* and *SLC25A4* genes have been linked with SANDO.

SCAE (SpinoCerebellar Ataxia – Epilepsy syndrome)

SCAE is a disorder similar to SANDO but with a higher frequency of migraine headaches and seizures (Winterthun *et al.*, 2005). Mutations in the *POLG* and *C10orf2* genes have been linked with SCAE.

MNGIE (Mitochondrial NeuroGastroIntestinal Encephalomyopathy)

MNGIE is an autosomal recessive disorder clinically characterized by onset between the second and fifth decades of life, PEO, gastrointestinal dysmotility (often pseudoobstruction), cachexia, diffuse leukoencephalopathy, peripheral neuropathy and early death. Abnormalities of mtDNA can include depletion, multiple deletions, and point mutations (Hirano *et al.*, 1994). Mutations in the *TYMP*, *RRM2B* and *POLG* genes have been linked with this phenotype. A brief description of the *TYMP* gene is given below.

i) TYMP (ECGF1)

The *TYMP* gene, responsible for MNGIE (Mitochondrial NeuroGastroIntestinal Encephalomyopathy), encodes the enzyme thymidine phosphorylase (TP), which is involved in pyrimidines catabolism. Defects of TP result in systemic accumulation of thymidine and deoxyuridine, which leads to deoxynucleotide pool imbalance and mtDNA instability, resulting in the presence of multiple deletions and partial depletion of muscle mtDNA (Nishino *et al.*, 1999).

The *TYMP* gene is located at chromosome 22, comprises 10 exons and spans 4.3 kb. The first pathogenic mutations were described in 1999 (Nishino *et al.*, 1999) and since then 80 mutations have been described as being associated with MNGIE.

1.3.1.2. Mitochondrial DNA depletion syndrome

Quantitative alterations are characterized by depletion of mtDNA. The mtDNA depletion syndrome (MDS) comprises a heterogeneous group of autosomal recessive disorders, all having the same molecular end result, low mtDNA amount in specific tissues. MDS are a group of rare and devastating diseases that manifest typically, although not exclusively, soon after birth, determining early death usually in infancy or early childhood. MDS differs from other MRC disorders, as most often it may manifest solely in a specific organ (most commonly muscle or liver) (Suomalainen *et al.*, 2010). However, it may occur that multiple organs, including heart, brain, and kidney are affected (Spinazzola *et al.*, 2007).

Three major clinical categories can be recognized however, the clinical phenotypes are heterogeneous, overlapping and ever expanding (Rötig and Poulton 2009; Suomalainen *et al.*, 2010).

Hepatocerebral MDS

Hepatocerebral MDS is most probably the most common variant of MDS. Onset of symptoms is between birth and 6 months; death usually occurs within one year of age. The most common symptoms and signs include persistent vomiting, failure to thrive, hypotonia and hypoglycemia associated with progressive neurological symptoms. Histological changes on liver biopsy include fatty degeneration, bile duct proliferation, fibrosis, and collapse of lobular architecture. Reduced COX histochemistry and combined deficiency of mtDNA encoded MRC complexes were found in the liver of a few patients.

A peculiar form of hepatocerebral MDS is AHS, an early onset, fatal disease, characterized by hepatic failure, intractable seizures, evolving into epilepsia partialis continua, and global neurological deterioration. The liver dysfunction is usually progressive as well, evolving from microvesicular steatosis with bile duct proliferation into cirrhosis and chronic liver failure. Mutations in *POLG* gene are a frequent cause of AHS and to date 55 mutations have been reported.

Mutations in the *POLG*, *C10orf2*, *DGUOK*, *MPV17* and *TK2* genes have been identified as being associated with hepatocerebral MDS.

i) DGUOK

Deoxyguanosine kinase is a 2-deoxyribonucleoside enzyme that catalyzes the first step of the mitochondrial deoxypurine salvage pathway, the phosphorylation of purine deoxyribonucleosides into the corresponding nucleotides deoxyguanosine and deoxyadenosine necessary for the maintenance of mitochondrial dNTPs pools (Copeland 2008; Spinazzola *et al.*, 2007). The typical phenotype of mutations in the *DGUOK* gene, on chromosome 2p13, is characterized by neonatal onset of progressive liver disease and feeding difficulties, usually with neurological dysfunction (hypotonia, nystagmus, and psychomotor retardation), by the age of 3 months. Peripheral neuropathy and renal tubulopathy have occasionally been reported (Rahman and Poulton, 2009). Depletion of mtDNA has been documented only in the liver and results in combined MRC deficiencies in the liver, whereas the amount of mtDNA is usually normal in muscle and fibroblasts. Histological analyses of the liver biopsy show variable findings, typically microvacuolar steatosis, cholestasis, fibrosis, and cirrhosis. In most cases, there is a rapidly progressive liver disease and neurological deterioration, with death occurring by the age of 12 months or shortly thereafter (Ji *et al.*, 2010). The first pathogenic mutations was reported in 2001 (Mandel *et al.*, 2001), since then more than 80 affected patients from approximately 50 families have been reported, and over 50 different *DGUOK* mutations have been identified (El-Hattab and Scaglia 2013). The infantile hepatocerebral form of MDS is the almost invariable clinical presentation. Genotype-phenotype correlation studies show that patients who harbor null mutations usually have early onset liver failure and significant neurological disease, including hypotonia, nystagmus, and psychomotor retardation, and death before two years of age. Patients carrying missense mutations usually have isolated liver disease, a better prognosis, and longer survival.

ii) MPV17

The *MPV17* gene is located on chromosome 2p23-p21 and encodes a IMM protein of unknown function but recently recognized as responsible for mtDNA depletion. The clinical presentation is that of severe liver failure, hypoglycemia, growth retardation, neurological symptoms, and multiple brain lesions during the first year of life (Wong *et al.*, 2007). Marked mtDNA depletion in the liver is the molecular hallmark associated with multiple defects of MRC complexes. Normal or mildly reduced levels of both mtDNA content and MRC enzyme activities were also found in muscle (Alberio *et al.*, 2007). Histological analyses of the liver have revealed swollen granular hepatocytes, microvesicular steatosis, and focal pericellular and periportal fibrosis. Since the first mutation was described in 2006 (Spinazzola *et al.*, 2006), 27 different mutations have been reported in infantile-onset hepatocerebral syndrome and in Navajo neurohepatopathy, which is an autosomal recessive multisystem disorder found in the Navajo of the southwestern United States (Copeland *et al.*, 2008). Three main subtypes are to be considered: infantile-onset (before 6 months) and childhood-onset (before 5 years) forms with hypoglycemic episodes and severe progressive liver dysfunction requiring liver transplant, and a "classic" form with moderate hepatopathy and progressive sensorimotor axonal neuropathy. The three forms are also associated with variable degrees of demyelination in both the central and the peripheral nervous system.

Myopathic MDS

Myopathic MDS onset of symptoms usually occur in the first year of life with feeding difficulty, failure to thrive, hypotonia, muscle weakness and occasionally PEO. Death is usually due to pulmonary insufficiency and infections, but some patients survive into their teens (Moraes *et al.*, 1991; Tritschler *et al.*, 1992). Muscle biopsy may show proliferation of mitochondria, which can increase with age, and patchy or diffuse COX deficiency. Biochemical defects of all mtDNA-related MRC complexes are always present in muscle mitochondria. Serum CK levels may be variably elevated (Spinazzola *et al.*, 2005).
Mutations in the *TK2* gene are the most frequently cause of myopathic MDS with 34 mutations reported however mutations in *POLG*, *RRM2B* and *DGUOK* genes have also been identified in myopathic MDS.

i) TK2

Thymidine kinase (TK2) is an intramitochondrial pyrimidine nucleoside kinase that phosphorylates dNTPs, such as: deoxythymidine, deoxycytidine, and deoxyuridine, thereby participating in the salvage pathway of deoxynucleotide synthesis in the mitochondria (Oskoui *et al.*, 2006). Mitochondrial dNTPs pools arise either through active transport of cytosolic dNTP or through salvage pathways. Both pathways are essential for the replication of mtDNA, since the mitochondrion is unable to synthesize dNTPs de novo. Mutations in the *TK2* gene on chromosome 16q22 affect primarily muscle tissue, with little or no effect on the liver, brain, heart, or skin. The typical manifestation of *TK2* mutations is a severe, rapidly progressing myopathy of infantile or childhood onset. The disease course is rapidly progressive, leading to respiratory failure and death in months or years, but milder phenotypes with slower progression and longer survival have been reported (Suomalainen *et al.*, 2010). Since the first mutation was described in 2001 (Saada *et al.*, 2001), approximately 38 different pathogenic mutations in *TK2* have been published so far, either as recessive homozygous or compound heterozygous mutations, and phenotypes may be explained by variable degrees of residual activity of the mutant enzymes.

Encephalomyopathic MDS

Encephalomyopathic MDS is characterized by infantile onset of hypotonia with severe psychomotor retardation, high lactate in blood, progressive neurologic deterioration, a hyperkinetic-dystonic movement disorder, external ophthalmoplegia, deafness, generalized seizures and variable renal tubular dysfunction. Brain MRI was suggestive of Leigh syndrome (Spinazzola *et al.*, 2007).

Mutations in the *RRM2B*, *SUCLA2*, *SUCLG1*, *TK2* and *C10orf2* genes have been identified as being associated with encephalomyopathic MDS.

i) RRM2B

The *RRM2B* gene on chromosome 8q23 encodes the small subunit of p53-inducible ribonucleotide reductase, a heterotetrameric enzyme responsible for the *de novo* conversion of ribonucleoside diphosphates into the corresponding deoxyribonucleoside diphosphates that are crucial for DNA synthesis (Bourdon *et al.*, 2007). The enzyme is the main regulator of nucleotide pools in the cytoplasm, and its small subunit is expressed in postmitotic cells, where it probably has a key function in maintaining the mitochondrial dNTPs pools for mtDNA synthesis. Mutations in *RRM2B* usually result in hypotonia, lactic acidosis, failure to thrive, and tubulopathy in the first months of life. The disease has a rapid progression and leads to death in few months. The associated complex phenotype suggests that the consequences of a defective mitochondrial dNTPs pools can vary dramatically depending on the residual amount of the functional enzyme. Recently, it has been shown that inactivating mutations in *RRM2B* also cause severe neonatal or infantile forms of mtDNA depletion, with profound reduction of mtDNA copy numbers in skeletal muscle (Bourdon *et al.*, 2007). The first pathogenic mutation was reported in 2007 (Bourdon *et al.*, 2007) and since then 29 mutations have been described from which 14 are associated with encephalomyopathic MDS, 11 with PEO, two with MNGIE and three with myopathic MDS.

ii) SUCLA2 and *SUCLG1*

Succinyl-CoA synthase is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinate and ATP or GTP from succinyl-CoA and ADP in the TCA cycle. This enzyme is made up of two subunits, α and β, encoded by *SUCLG1* on chromosome 2p11 and *SUCLA2* on 13q12, respectively. Mutations in *SUCLA2* and *SUCLG1* cause an encephalomyopathic form of infantile mtDNA depletion syndrome, but *SUCLG1* can also cause a very severe disorder with antenatal dysmorphisms, neonatal metabolic crisis, and early death, probably depending on the residual amount of the protein (Morava *et al.*, 2009; Rouzier *et al.*, 2010). An useful diagnostic clue in succinyl-CoA synthase disorders of succinyl-CoA synthase is a ‘‘mildly’’ elevated urinary methylmalonic acid (MMA), which is detected in all patients, and the presence of TCA intermediates (methylcitrate, lactate, carnitine esters, 3-hydroxyisovaleric acid) in most cases. Some patients die early (sudden infant death syndrome), but some of them have a longer survival. The clinical features of patients with mutations in these genes include early childhood hypotonia, developmental delay, and almost invariably, progressive dystonia and sensorineural deafness. *SUCLA2* and *SUCLG1* mutations seem to disrupt an association between succinyl-CoA synthase and mitochondrial nucleoside diphosphate kinase, resulting in an unbalanced mitochondrial dNTP pool and eventually, mtDNA depletion in muscle. The first pathogenic mutations were reported in 2005 (Elpeleg *et al.*, 2005) and 2007 (Ostergaard *et al.*, 2007) in *SUCLA2* and *SUCLG1*, respectively, and since then seven and twelve mutations have been reported as associated with encephalomyopathic MDS.

1.4. Treatment of mitochondrial disorders

There is no effective treatment for mitochondrial disorders, and current clinical management is focused on treating complications or alleviating symptoms. Therapy is inadequate and mostly palliative, but promising new approaches are being identified.

Treatments used to modify the underlying disease process fall into three major categories: i) symptomatic therapy (palliative therapy is dictated by good medical practice and includes anticonvulsant medication, control of endocrine dysfunction, surgical procedures, etc.), ii) pharmacological therapy (aimed to remove toxic metabolites, supplementation with vitamins and cofactors to “strengthen” the MRC, scavenging excessive ROS, etc.) and iii) gene therapy.

1.4.1. Symptomatic therapy

Lack of a cure for mitochondrial disorders does not exclude treatment and symptomatic therapy can be very effective in these patients.

Seizures usually respond to conventional anticonvulsants. However, valproic acid should be used with caution and in association with L-carnitine, because it inhibits carnitine uptake (Tein *et al.*, 1993). Valproic acid is especially dangerous in children with AHS, a hepatocerebral disorder with mtDNA depletion due to mutations in the gene encoding *POLG*. Seizures are often difficult to manage in AHS and valproic acid can trigger acute hepatic failure (Bicknese *et al.*, 1992).

In patients with PEO, severe ptosis can be ameliorated by selective surgery, at least transiently. Congenital cataracts are also treated surgically.

Diabetes mellitus, whether insulin-dependent or not, responds to dietary or pharmacological therapy. The use of growth hormone in children with growth retardation is controversial because the increased metabolic demands may be ill tolerated by an already metabolically challenged patient. In children with impaired growth due to feeding problems, recurrent vomiting, or severe gastroesophageal reflux, useful surgical procedures include percutaneous endoscopic gastrostomy or fundoplication.

Timely placement of a pacemaker can be life-saving in patients with KSS and blocks of cardiac conduction. Heart transplantation is controversial in patients with cardiomyopathy and multisystemic disorders. However, when cardiac involvement is the predominant or exclusive problem, cardiac transplantation is justified (Tranchant *et al.*, 1993; Bohlega *et al.*, 1996; Santorelli *et al.*, 2002).

Neurosensory hearing loss can be alleviated by cochlear implants (Sue *et al.*, 1998).

Liver failure, often associated with the mtDNA depletion syndrome, may benefit from liver transplantation, especially if other organs seem to be spared (Dubern *et al.*, 2011; Salvati *et al.*, 2002).

Recurrent myoglobinuria is seen in patients with primary CoQ10 deficiency or with mutations in mtDNA protein-coding gene (Ogasahara *et al.*, 1989; Sobreira *et al.*, 1997; Di Giovanni *et al.*, 2001). Patients with CoQ10 deficiency benefit from CoQ10 supplementation. During acute episodes, all patients should be rehydrated and subjected to renal dialysis when myoglobinuria is complicated by renal failure.

Psychotropic drugs are effective in patients with psychiatric symptoms (especially depression), which are not infrequent, and sometimes isolated, in mitochondrial patients (DiMauro *et al.*, 2013).

Physical, occupational, respiratory, and speech therapy are of great value in accelerating recovery from stroke in patients with MELAS and in aiding patients with other CNS problems (ataxia, dysarthria, dysphagia, spasticity, paralysis) (Millhouse-Flourie et al., 2004).

Exercise and physical therapy had to be considered in patients with mitochondrial diseases due to the fact that these disorders are frequently associated with mitochondrial proliferation and that sustained exercise also is associated with increased of mitochondrial content in muscle. Inactivity in patients with mitochondrial diseases and exercise intolerance is often excused for fear of causing muscle damage, but it only increases deconditioning. Instead, aerobic training in these patients has remarkable effects, increasing work and oxidation capacity.

In baseline studies of a larger number of subjects with mitochondrial myopathy and normal controls, it was found that the percentage of mutant mtDNA correlates indirectly with exercise tolerance. Further independent studies have confirmed these benefits in subjects with mtDNA deletions or point mutations, including MELAS. One of these studies showed that total mtDNA increases after exercise in subjects with mtDNA mutations (but not normal subjects) without significantly altering the ratio of normal/mutant mtDNA (Kerr *et al.*, 2010).

1.4.2 Pharmacological therapy

Elimination of noxious compounds

One of the therapeutic approaches to mitochondrial disease is to eliminate the noxious compounds that accumulate in these disorders. To decrease brain lactate in patients with MELAS, dichloroacetate was used, which keept pyruvate dehydrogenase in the active form and favours lactate oxidation. However, this agent had unacceptable neurotoxicity, leading to treatment discontinuation (Kaufmann *et al.*, 2006). A more promising 'detoxifying therapy' seems to be allogeneic haematopoetic stem-cell transplantation (AHSCT) aimed at restoring sufficient thymidine phosphorylase activity in patients with MNGIE to normalize circulating, toxic levels of thymidine and deoxyuridine (Hirano *et al.*, 2012). A safety study of AHSCT in patients with MNGIE is under way.

Administration of Electron Acceptors

CoQ10 is both a component of the electron-transport chain and an effective oxygen radical scavenger. Its dual function, in addition to its safety, make CoQ10 one of the most widely used supplements in mitochondrial diseases (DiMauro *et al.*, 2006a).

There are several reports of the beneficial effect of CoQ10 in mitochondrial diseases, usually as a component of a ''cocktail'' that also includes L-carnitine, vitamin B complex, vitamin C and vitamin K1 (Marriage *et al.*, 2004). CoQ10 supplementation is mandatory and at times even life-saving in the primary CoQ10 deficiency syndromes. Although the response to CoQ10 administration seems to vary in different patients, probably depending on the mutant biosynthetic gene, supplementation with high CoQ10 doses should be tried in all patients (Schon *et al.*, 2010).

Two synthetic analogues of CoQ10, idebenone and parabenzoquinone, seem more promising. Idebenone, a short-chain benzoquinone, has shown positive results in two studies of patients with LHON (Carelli *et al.*, 2011; Klopstock *et al.*, 2011). The second compound, a parabenzoquinone named EPI-743, has so far been tested only in open-label studies. This compound reversed vision loss in four of five patients with LHON (Sadun *et al.*, 2012), produced clinical improvement in 12 children with various mitochondrial disorders (Enns *et al.*, 2012), and arrested or reversed disease progression in 13 children with genetically proven Leigh syndrome (Martinelli *et al.*, 2012).

Administration of Vitamin and Cofactors

Various "cocktails" of vitamins and cofactors have been used–and are still used in patients with mitochondrial encephalomyopathies, including riboflavin (vitamin B2), thiamine (vitamin B1), folic acid, CoQ10, L-carnitine, and creatine. All of these are natural compounds and presumably harmless at the doses used. Some, such as riboflavin (an important co-factor for the function of numerous dehydrogenases) are components of the MRC, but there is no evidence that they are decreased in primary mitochondrial diseases (DiMauro *et al.*, 2006a). Others appear to be decreased in certain conditions; for example, folic acid was lower than normal in the blood and CSF of patients with KSS. Still others are decreased secondarily; free carnitine tends to be lower than normal in the blood of patients with MRC defects, whereas esterified carnitine tends to be increased. This shift may reflect a partial impairment of β-oxidation, whose reducing equivalents enter the MRC at the level of CoQ10 through the action of the electron transfer flavoprotein.

Creatine monohydrate supplementation has been used with the goal of improving ATP synthesis and mean power output, but two randomized studies in patients with mitochondrial diseases came to different conclusions: a smaller cohort of severely affected patients improved, whereas a larger cohort of less severely affected patients did not (DiMauro *et al.*, 2006a).

Koga and coworkers found that plasma concentrations of both citrulline and arginine were decreased in MELAS patients during and between stroke-like episodes (Koga *et al.*, 2005). They also found that intravenous administration of l-arginine (0.5 g/kg) during the acute phase improved all stroke-like symptoms, whereas interictal oral administration (0,15–0,30g/kg daily) diminished both frequency and severity of strokes.

In vitro studies have revealed a potentially useful therapeutic approach to a fatal infantile form of encephalocardiomyopathy associated with COX deficiency and due to mutations in the *SCO2* gene, which encodes a COX-assembly protein needed for the insertion of copper into the holoenzyme. When copper was added to the medium of cultured COX-deficient myoblasts harboring *SCO2* mutations, COX activity was restored (DiMauro *et al.*, 2006a).

Finally, mtDNA depletion could be prevented by the addition of defective products [deoxyguanosine monophosphate (dGMP) and deoxyadenosine monophosphate (dAMP)] of the enzyme, deoxyguanosine kinase, in fibroblasts from a patient with hepatocerebral syndrome and a homozygous nonsense mutation in the *DGUOK* gene (Taanman *et al.*, 2003). As the investigators pointed out, further studies in animal models of deoxyguanosine kinase deficiency are needed to establish the uptake, therapeutic value, and possible side effects of deoxynucleotide administration.

The rationale for using these compounds is two fold: some of them are actually decreased in patients, either primarily or secondarily, whereas others are considered “neuroprotective” in that they may favor ATP production and counteract excitotoxicity, calcium-activated proteolysis, free radical generation, or apoptosis (DiMauro *et al.*, 2013).

Administration of Oxygen Radical Scavengers

Defects of the MRC have detrimental effects that go beyond impairing ATP production and include altered intracellular calcium buffering, excessive production of ROS, and promoting apoptosis (Hays *et al.*, 2006).

ROS are normal toxic secondary products of the MRC. They derive from the direct reaction of electrons in the MRC with O_2 to generate superoxide anion (O_2^-), which can be detoxified by mitochondrial manganese superoxide dismutase (MnSOD) with liberation of hydrogen peroxide (H_2O_2). In the presence of transition metals, H_2O_2 can be reduced to a very toxic hydroxyl radical ($^{\cdot}OH$). However, H_2O_2 can also be detoxified either through reduction to water by glutathione peroxidase or through conversion to O_2 and H_2O by catalase.

Defects of the MRC increase the production of ROS, which, in turn, damage cell membranes through lipid peroxidation and accelerate the already high mutation rate of mtDNA, creating a vicious cycle.

The concept of “oxidative stress” has taken center stage in the pathogenesis not only of “primary” mitochondrial disorders (i.e., defects of the MRC) but also of late-onset neurodegenerative diseases. In an attempt to quench the effects of ROS, several oxygen radical scavengers have been utilized, including vitamin E, CoQ10, idebenone, glutathione, and dihydrolipoate. Although no formal large-scale clinical trial has yet been conducted, glutathione administration appears promising because glutathione concentration was markedly decreased in muscle specimens from patients with isolated or combined respiratory-chain defects.

CoQ10 is widely used in primary mitochondrial diseases, and the plethora of generally positive anecdotal data together with the lack of negative side effects has contributed to its popularity. CoQ10 and its analog, idebenone, have also been used widely in the treatment of neurodegenerative disorders (DiMauro *et al.*, 2006b).

Enhance of mitochondrial biogenesis

The enhancement of overall mitochondrial biogenesis has emerged as an exciting therapeutic prospect in the last years, through activation of the transcriptional co-activator PGC-1α. The advantage over disease-induced mitochondrial proliferation, which favours mutated mtDNAs, is that pharmacological upregulation of mitochondrial biosynthesis increases numbers of all mtDNA molecules, allowing wild-type genomes to compensate for mutated ones. Encouraging results have been obtained in four mouse models with COX deficiency (DiMauro *et al.*, 2013).

Recently, a large number of compounds with therapeutic potential have been described. These include polyphenolic phytochemicals such as resveratrol, grape seed extract, green tea extract and genistein. Resveratrol is a natural phytoalexin found in a wide variety of plant species, including grapes. Among its numerous properties, resveratrol has been reported to have anti-oxidant activities and to activate the genetic expression of key genes in energy metabolism such as PGC-1α. Resveratrol and grape seed extract (a proanthocyanidin) were demonstrated to have beneficial effects on mitochondrial function in several experimental models (Lagouge *et al.*, 2006). Green tea polyphenols attenuated mitochondrial dysfunction in glucose deprived glial cell cultures. Genistein is a soy derived isoflavone which has been evaluated in substrate reduction therapy for mucopolysaccharidoses and was also reported to induce mitochondrial biogenesis (Kloska *et al.*, 2011; Rasbach *et al.*, 2008).

In addition to polyphenols, other substances such as compounds enhancing energy metabolism, antioxidants and chemical chaperones are potentially beneficial. Representative of this type are AICAR, oltipraz, bezafibrate and sodium phenylbutyrate (Golubitzky *et al.*, 2011).

AICAR is a pharmacological activator of AMP activated protein kinase (AMPK). This heterotrimeric protein complex plays a key role in the regulation of energy homeostasis. The kinase is activated by an elevated AMP/ATP ratio caused by cellular and environmental stress, such as heat shock, hypoxia and ischemia. AMPK regulates energy expenditure by modulating $NADH^+$ dependent-type III deacetylase SIRT1, resulting in the deacetylation of downstream targets including PGC-1α forkhead box O1 and 3 transcription factors (Canto *et al.*, 2009).

Oltipraz is a 1,2-dithiole-3-thione compound with antioxidant properties. Oltipraz has also been demonstrated to reduce apoptosis in cells with chemically inhibited CI by exerting its cytoprotective effect though AMPK (Golubitzky *et al.*, 2011).

Bezafibrate is an agonist of PPARs stimulating oxidative metabolism and has a documented positive effect on mitochondria. On the other hand, fenofibrate was reported to have a negative effect on CI (Wenz *et al.*, 2010).

Sodium phenyl butyrate is a histone deacetylase (HDAC) inhibitor, affecting protein phosphorylation and relief of endoplasmic reticulum stress. Although the mechanism of action of this compound is poorly defined, it has been found to be beneficial in a number of diseases including cancer, neurodegenerative diseases and metabolic diseases (Cudkowicz *et al.*, 2009).

Finally, Bendavia (MTP-131) is a novel therapeutic agent that preserves mitochondrial integrity and ATP synthesis, while reducing ROS production. This compound acts via a unique mechanism, selectively targeting the IMM by interacting with cardiolipin to facilitate flux through the electron transport chain, providing more efficient electron flow. Importantly, Bendavia does not seem to scavenge ROS and does not require a transmembrane potential to be efficient, which differentiate it from other therapeutic approaches that target mitochondria (Chen *et al.*, 2011).

All of the aforementioned compounds have been documented to exert positive effects in OXPHOS deficient patient's cells.

1.4.3. Gene therapy

For nDNA-related mitochondrial disorders, 'classic' gene therapy is an option, but well-known issues are associated with this approach, including choice of appropriate viral or nonviral vectors, delivery to the affected tissues, and potential immunological reactions. Nonetheless, adeno-associated virus-mediated gene transfer has proven successful in two animal models: the *Ant1* mutant mouse (Flierl *et al.*, 2005) and a mouse model of ethylmalonic encephalomyopathy (Di Meo *et al.*, 2012).

For mitochondrial diseases due to mutations in mtDNA, the problems are more complex because of polyplasmy and heteroplasmy and because no investigators have so far been able to transfect DNA into mitochondria in a heritable manner (DiMauro *et al.*, 2006a).

Shifting of heteroplasmy

For mtDNA-related disorders, an obvious but challenging goal is to shift heteroplasmy to lower the mutation load to subthreshold levels. Several strategies have been attempted in cybrid cell lines, often with good results. When deprived of glucose and exposed to ketogenic media, cybrids harbouring single mtDNA deletions shifted their heteroplasmy level and recovered mitochondrial function, probably through selective mitochondrial autophagy (mitophagy) (Gilkerson *et al.*, 2012). Suomalainen's group recently used a ketogenic diet to rescue metabolic function in a mouse model of late-onset mitochondrial myopathy due to deletions of mtDNA but, interestingly, the metabolic improvement was not accompanied by any obvious reduction in mutation load (Ahola-Erkkila *et al.*, 2010). This discrepancy might be explained, however, by the possibility that instead of reducing mutation load, the ketogenic stress "normalized" the distribution of the mutation so that cells contained more mitochondria below the threshold for dysfunction (Schon *et al.*, 2010).

A genetic approach to heteroplasmic shifting involves use of restriction endonucleases to eliminate specific pathogenic mutations. Dormant stem cells in skeletal muscle (satellite cells) have fewer mtDNA mutations than do adult muscle fibres, and muscle regeneration after induced myonecrosis was used to shift heteroplasmy in a patient with strabismus. For example, the T to G mutation at nt-8993 in the *MT-ATP6* gene that causes MILS and/or NARP creates a novel and unique *SmaI* site in human mtDNA. Because WT mtDNA has no *SmaI* sites, if functioning *SmaI* could be targeted to mitochondria in cells from heteroplasmic NARP/MILS patients, it would be possible to selectively cleave (and thereby destroy) the mutated mtDNAs while leaving the WT mtDNAs intact (Figure 12).

In 2002, Tanaka and colleagues demonstrated that such approach is possible. Although this strategy would only work for a mutation that generates a unique restriction site absent in wt mtDNA, the precedent for the importation of a nuclease into mitochondria has been set.

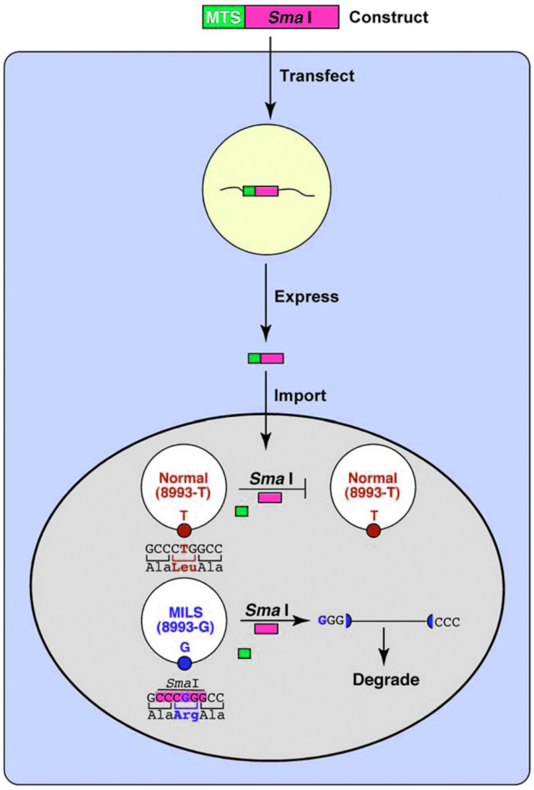


Figure 12 - Mitochondrial importation of a restriction endonuclease to cleave mutated mtDNAs selectively. The example shown is of the importation of *SmaI* (pink rectangle) to eliminate the T→G mutation at mtDNA position 8993 (p.L156R mutation in ATP6) associated with NARP/MILS. Following the transfection of the construct into the cell (blue), mitochondrially targeted *SmaI* expressed from the nucleus (yellow) is targeted to the organelle (gray) via an N-terminal mitochondrial targeting sequence (MTS; green rectangle) that is cleaved from the precursor polypeptide following import. Only mutated mtDNAs are cleaved and then degraded, leaving the normal mtDNAs intact (figure adapted from Schon *et al.*, 2010).

Fighting against ROS Accumulation

A genetic approach to scavenging ROS proved successful in transgenic mice. First, it was documented that the complex I deficiency brought about by expression of an anti-NDUFA1 ribozyme (NDUFA1 is a catalytic subunit of complex I) induced lesions of the retina and optic nerve similar to those of LHON in humans. Second, it was shown that downregulation of MnSOD by expression of anti-SOD2 ribozyme in mice also induced LHON-like lesions. These data established that excessive production of ROS damaged the retina and the optic nerve. When the investigators coexpressed anti-NDUFA1 rybozyme (the poison) and SOD2 (the antidote) in the same mice, the lesions were not produced, thus documenting the powerful therapeutic action of SOD scavenging (DiMauro *et al.*, 2006b).

Stem Cell Therapy

Promise for stem cell therapy in mtDNA-related diseases is offered by a study documenting that mitochondria can migrate from cell to adjacent cell. Coculturing human rho0 cells with normal osteoblastoma cells or fibroblasts resulted in rescue of the mitochondrial function of rho0 cells due to transfer of normal mitochondria, which occurred in the absence of cell fusion (Spees *et al.*, 2006).

Cytoplasmic transfer

For mtDNA-related diseases, many of which are devastating and undiagnosable before birth, the ultimate goal is to prevent their occurrence altogether via cytoplasmic transfer. In this approach, the nucleus of an *in vitro*-fertilized oocyte from a carrier is transferred to an enucleated oocyte from a normal donor: the embryo will have the nDNA of the biological parents but the mtDNA of a normal mitochondrial donor (Figure 13). This approach would result in a mitochondrially normal child carrying the nuclear traits of both parents. The feasibility of cytoplasmic transfer has now been demonstrated by the Newcastle group in the UK (Craven *et al.*, 2010), and a variant of this approach was recently used in the US to produce two "transmitochondrial" rhesus monkeys (Tachibana *et al.*, 2009). Of course, the ethics of cytoplasmic transfer would need to be addressed before this type of germline gene therapy could be undertaken (Rubenstein *et al.*, 2005).

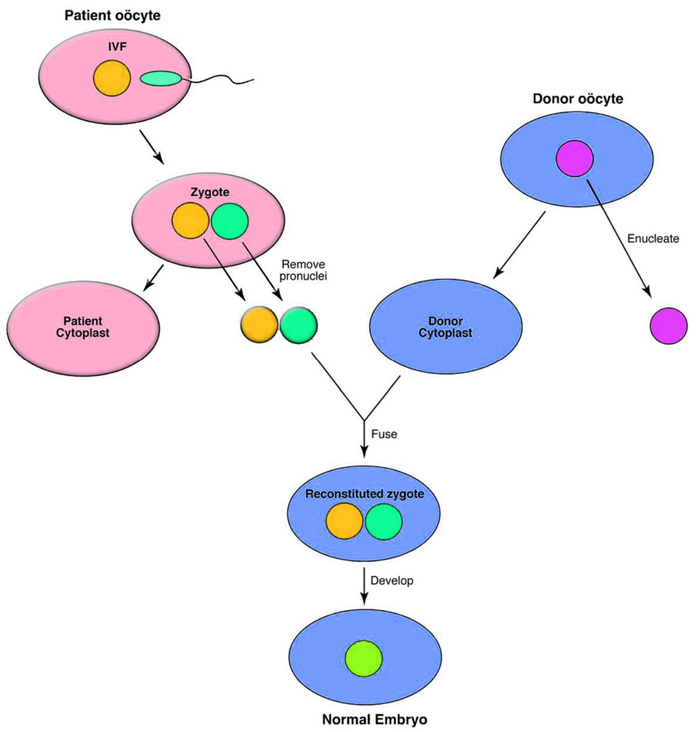


Figure 13 - Cytoplasmic transfer. Following in vitro fertilization, the haploid parental pronuclei (i.e. the normal pronucleus from the ooplasm of a woman carrying a mtDNA mutation [pink] and the normal pronucleus from the father [light green]) are transferred to an enucleated donor oocyte containing normal mtDNAs (blue); following fusion of the pronuclei, the embryo now contains a normal diploid nucleus from the parents (green) and normal mtDNA from the cytoplasmic donor, and can be implanted into the mother's uterus figure adapted from Schon *et al.*, 2010).

1.5. Genetic counseling

Prenatal diagnosis for tRNA point mutations, including the common ones associated with MELAS and MERRF, is practically impossible because of two concerns. First, the mutation load in amniocytes or chorionic villi does not necessarily correspond to that of other fetal tissues. Second, mutation load measured in prenatal samples may shift *in utero* or after birth due to mitotic segregation.

At the other end of the spectrum, large-scale deletions of mtDNA as a rule are neither inherited nor transmitted. They probably arise *de novo* in oogenesis or in early embryogenesis, or, when they are present in a fertilized oocyte, are unlikely to slip through the bottleneck between ovum and embryo that allows only a small minority of maternal mtDNAs to populate the fetus. However, in counseling a woman who harbors a large-scale mtDNA deletion, the possibility of transmission should not be excluded completely. There is good evidence that mutations in *ATPase 6* associated with NARP/MILS do not show tissue- or age-related variations, thus making prenatal diagnosis feasible for parents who have lost a child to maternally inherited Leigh’s syndrome. However, once medium-low levels of mutated genomes are found in chorion villi, it is not practicable to assure parents on the future safety of their offspring.

The rapid progress in our molecular knowledge of nDNA-related defects of the MRC is improving genetic counseling and making prenatal diagnosis an option for families with fatal infantile conditions, such as Leigh’s syndrome or mtDNA depletion syndromes (DiMauro *et al.*, 2004).

CHAPTER II

AIMS

AIMS

The mitochondrial and nuclear genomes exert a dual genetic control on the OXPHOS and associated systems. Diseases caused by nuclear genes, that affect mtDNA stability are an interesting group of mitochondrial disorders, involving both cellular genomes. In these pathologies, a primary nuclear gene defect causes either qualitative (mtDNA multiple deletions) or quantitative (mtDNA depletion) molecular abnormalities, which leads to tissue dysfunction.

The overall aim of this project was to study patients with nuclear-mitochondrial intergenomic communication using the following approach:

i) to identify patients with mtDNA multiple deletions and/or mtDNA depletion by real-time qPCR.
ii) to perform mutation screening of nuclear genes associated with disorders of cross-talk in the previous identified patients.
iii) to expand the number of identified mutations in these genes.
iv) to set up a number of functional assays to appreciate the efficacy/toxicity of standard and new therapeutic agents in cross-talk disorders.

The current project should improve the study of these patients in our country, by providing a complete molecular diagnosis approach to find the causative nuclear gene mutations and by contributing to the development of novel therapeutic strategies against these disorders.

CHAPTER III

PATIENTS AND METHODS

PATIENTS AND METHODS

3.1. Patients

The Laboratory of Mitochondrial Pathology at the Genetic and Metabolism Unit of the Genetic Department from the Portuguese National Institute of Health (INSA, IP) is a reference center for mitochondrial disorders of oxidative metabolism in Portugal since 1993.

In the past 20 years, the laboratory has received more than 2000 samples (muscle, liver, fibroblasts, and blood) to study a possible defect causing mitochondrial disease. Patients had been referred from hospitals from all over the Country and overseas (Italy, Brazil and The Netherlands).

For this study a total of 90 samples (52 muscle, 10 liver, 5 fibroblasts and 23 blood samples) were selected to investigate a possible defect of intergenomic communication (multiple deletions or mtDNA depletion). According to standard clinical and biochemical criteria, patients for this study (84 paediatric and 6 adults) were divided into three major groups (Figure 14):

i) The first group consisted of 26 individuals with a hepatocerebral presentation. The major clinical criteria to be included in this group were liver diseases (cholestasis, hepatomegaly, altered hepatic enzymes or liver failure) with lactic acidaemia and neurological dysfunction (including hypotonia, developmental delay or cerebral atrophy). Muscle or liver samples were available from 4 and 10 patients, respectively. Eligible patients showed either altered activities of the MRC complexes except for complex II, or generalized deficiencies of OXPHOS complexes, or normal activities in muscle in spite of a severe liver pathology. Solid tissues from 12 patients were not available, but were included in the genetic study due to a clinical history highly suggestive of the hepatocerebral form of MDS. Total DNA was obtained in these cases from blood.

ii) The second group was made of 22 individuals with an encephalomyopathic presentation. The major criterion to be included in this group was occurrence of “mild” methylmalonic aciduria with normal homocistinuria and the presence of neurological symptoms such as hypotonia, developmental delay, sensoneuronal deafness and clinical features suggestive of Leigh, or Leigh-like, syndrome. In addition, some patients showed lactic acidaemia and increased succinylcarnitine (C4-dicarboxylic carnitine - C4DC) in serum. Muscle tissue was available only from 9 patients, and DNA of the remaining 13 cases was studied from blood in 10 patients, or cultured skin fibroblasts in three cases.

iii) The third group was made of 42 individuals showing a purely myopathic presentation of a mitochondrial disorder. Feeding difficulties, failure to thrive, hypotonia, muscle weakness or wasting and occasionally ptosis and PEO were the major clinical criteria to be included in this group. Muscle tissue from 39 patients and fibroblasts from two were available, whereas in the remaining patient blood DNA was analyzed.

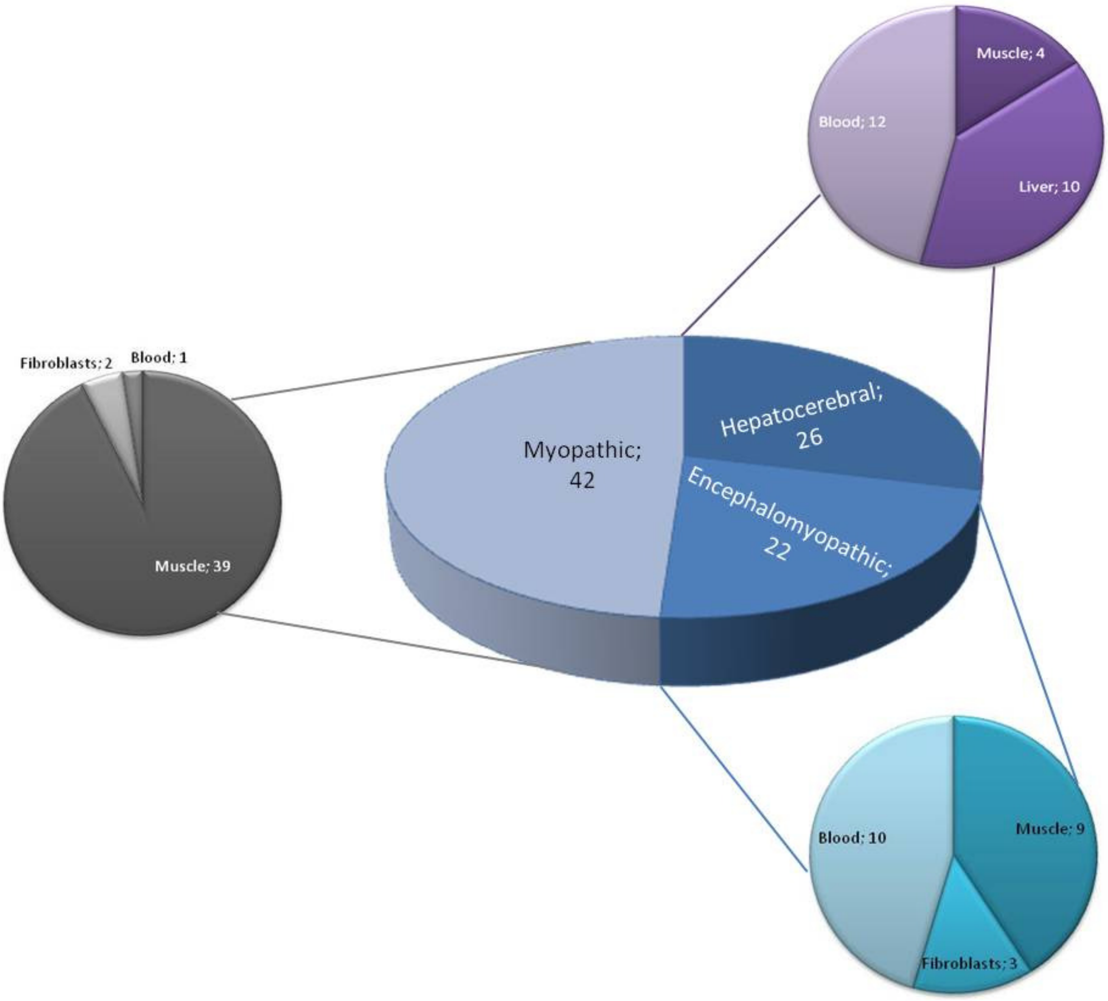


Figure 14 – Schematic clustering of the samples selected to investigate a possible defect of intergenomic communication. A group of patients (N=90) was distributed in three different categories of clinical presentation.

Before considering these patients to start molecular investigations it is important to obtain the appropriate biochemical and/or clinical information. Suspicion of intergenomic communication disorders arising from clinical presentation may range from well defined syndromes to unspecific multisystemic phenotype, where neurological involvement is usually present. Biochemical data, such as lactate, pyruvate, alanine, organic acid profiles as well as neuroimaging findings are also important clues for the diagnosis of these disorders. Establishing a specific diagnosis in a patient with suspected Mendelian disease is challenging and requires integration of clinical assessments, family history, biochemical testing and histopathological examination.

Thus, to select patients eligible for this study, among a total of 90 cases, the relative mtDNA/nDNA ratio by real-time qPCR was analyzed in samples from solid tissues in order to identify patients with mtDNA depletion or multiple deletions. Regarding patients with mtDNA depletion, we defined “primary” or a “secondary” mtDNA depletion when a significantly low level of mtDNA (that is, <30% range of control samples) or a moderate reduction (that is, 40-60% of the control range) was detected, respectively.

From the 90 initial cases, 47 were considered eligible for further investigations using an algorithm that favors prioritization in molecular diagnosis (Figure 15).

A molecular study was performed in 27 patients with mtDNA quantitative/qualitative alterations, and in 20 patients in whom DNA could be obtained only from blood, taking into account their clinical and biochemical features. Table 2 summarizes the clinical and biochemical data of these 47 eligible patients.

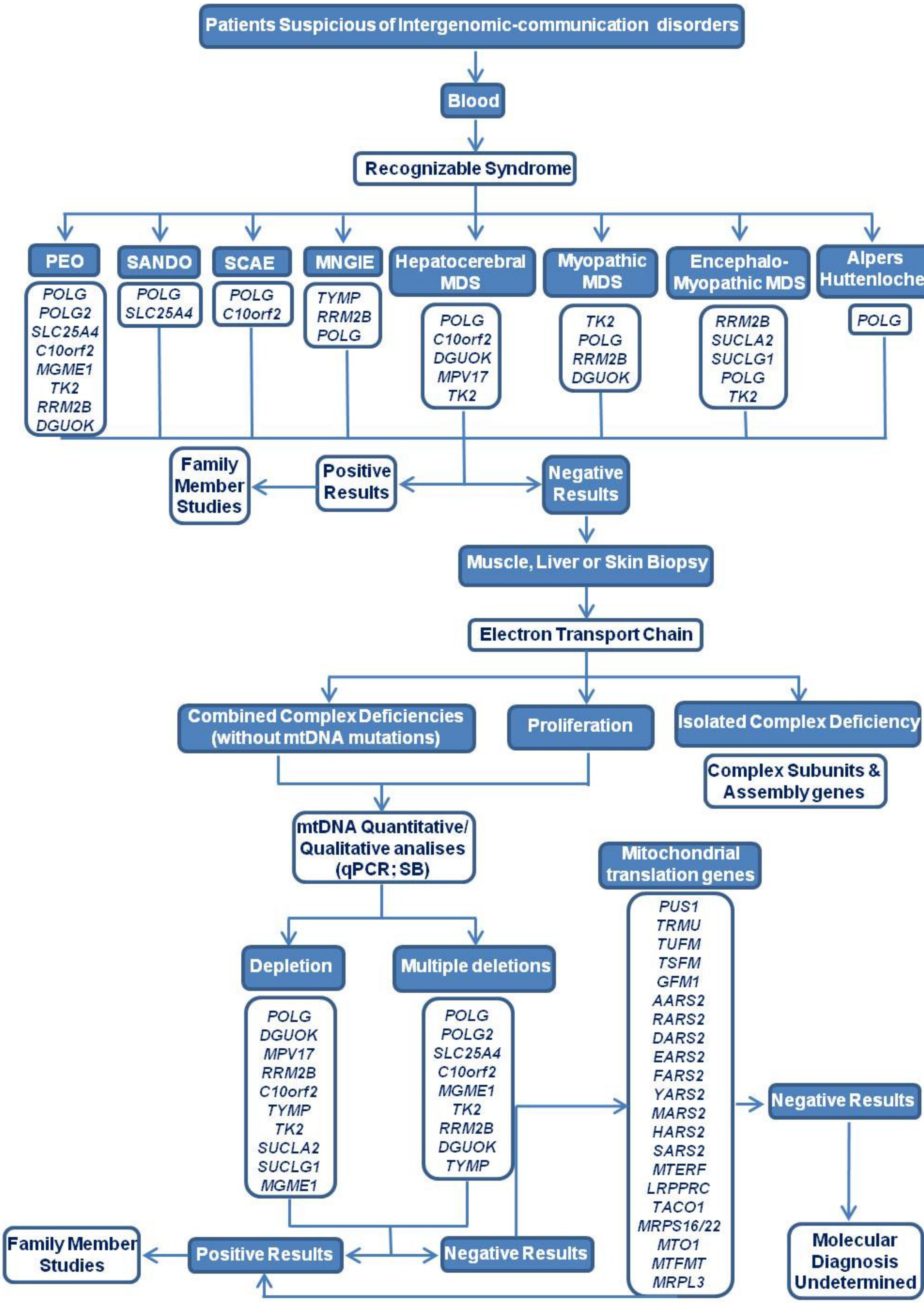


Figure 15 - Diagnostic algorithm for investigate suspicious patients of intergenomic communication disorders.

Table 2 – Clinical and biochemical data of the 47 eligible patients for this study.

Patient	Sex	Diagnostic age	Country	Specimen	Clinical and Biochemical data
Hepatocerebral form					
1†	M	29 Days	Pt	Mus	Severe acidosis; Cholestasis; Mutiorganic/ Liver failure; Cardio-respiratory arrest
2†	F	1 Mo	Pt	Bld	Coagulopathy; Hepatopathy; Anemia.
3	M	2 Mo	Pt	Bld	Hepatopathy with hepatic fibrosis; Ascite; Cardiac Ventricular Hypertrophy; Severe malnutrition; Neonatal Cholestasis; Hypertrophic Cardiomyopathy; Muscle atrophy.
4	M	10 Mo	Pt	Bld	Hepatic cytolysis; Insulin-dependent diabetes; Liver alterations; Relevant family history (consanguinity and brother with a similar picture).
5†	F	5 yrs	Br	Bld	Myoclonus; Seizures with left hemiparesis; Hypotonia; Difficulty in walking and in the balance maintaining; Developmental delay; Metabolic acidosis; Liver failure; Involuntary eye movements.
6	M	1 Mo	Pt	Bld	Cholestasis; Myoclonies.
7	M	1 yrs	Pt	Bld	Unexplained cholestasis in the first months of life; Changes in brain imaging; Macrocephaly; Cardiac and pulmonary alterations (pulmonary valve stenosis).
8	F	15 Mo	Pt	Liv	Liver failure.
9†	M	9 Mo	It	Liv	Hypotonia; *status epilepticus*; MRI with basal ganglia changes and atrophy; Abnormal liver function suggesting AHS; Liver failure.
10	M	1 Mo	Pt	Bld	Liver failure.
11	M	1 Mo	Pt	Bld	Neonatal hemochromatosis; Liver failure, Hypotonia; Feeeding difficulties; Hyperlactacidemia; Brother (P11a) deceased during neonatal period.
12	M	1,5 Mo	Pt	Bld	Liver failure.
13	M	3 Mo	Pt	Bld	Liver failure.
14†	M	2 Mo	Pt	Liv	Acute liver failure; Elevated levels of transaminases; thrombocytopenia.
15†	M	1 Mo	Br	Liv	Neonatal hypoglycemia; Digestive bleeding; Sepsis; Elevated levels of total and free serum bilirubin and transaminases. Hyperlactacidemia; Failure to thrive; Hypotonia; Nystagmus; Cirrhotic ascites; Sister (P15a) deceased with hepatic insufficiency.
16†	M	4 yrs	Nth	Liv	Seizures; hypoglycemia; Liver Failure; Steatosis; Hyperlactacidemia.

17	M	11 yrs	Pt	Bld	Hypotonia; Failure to thrive; Liver failure
18	M	13 yrs	Nth	Liv	Myoclonic epilepsy; Headache and bilateral high signal lesions of the occipital horns of the ventricles. Liver failure after treatment with valproate; Liver transplantation; Multi-organ involvement.
Encephalomyopathic form					
19	F	15 Days	Pt	Bld	Lactic acidaemia; Hypotonia; Developmental delay.
20	M	4 yrs	It	Fibr	Rapidly progressive cerebral and ponto-cerebellar atrophy with intractable epilepsy; Hyperlactacidemia; Neonatal Hypotonia; Intractable seizures; Frequent myoclonic jerks in upper limbs.
21	F	1 Mo	Pt	Bld	"Mild" methylmalonic aciduria with normal homocistinuria; Increased C4DC.
22	M	3,5 yrs	Pt	Bld	Developmental delay; Hyperlactacidemia; increased blood C4DC.
23	M	3 Mo	Pt	Bld	Hypotonia; Frequent vomiting; Sepsis; "Mild" methylmalonic aciduria with normal homocistinuria.
24	M	4 Mo	Pt	Bld	Hypotonia; "Mild" methylmalonic aciduria with normal homocistinuria; Dysmorphism.
25	M	1 yrs	Pt	Bld	Hypotonia; "Mild" methylmalonic aciduria with normal homocistinuria.
26	M	9 Mo	Pt	Bld	Hypotonia; Irritability; "Mild" methylmalonic aciduria with normal homocistinuria.
27	M	17 Mo	Pt	Fibr	Hypotonia; Failure to thrive; developmental delay; Weight loss during a gastroenteritis crisis; dysmorphisms; Muscular atrophy; Deafness; Hyperlactacidemia; moderate urinary excretion of MMA and elevated C4DC.
28	F	1 Mo	Pt	Bld	Failure to thrive; developmental delay; elevated C4DC.
29	M	3 yrs	Pt	Mus	Failure to thrive; Hypotonia; moderate urinary excretion of MMA with normal homocistinuria.
30	M	4 Mo	Pt	Bld	Hypotonia;Dysmorphisms; hyperlactacidemia moderate urinary excretion of MMA with normal homocistinuria.
31	F	1 Mo	Pt	Mus	Hyperlactacidemia; Failure to thrive; Hypotonia.
32	M	2 yrs	Pt	Mus	Developmental delay; Hyperlactacidemia; Progressive neurologic findings including, Strabismus; Ataxia.
33	M	49 yrs	Pt	Mus	Progressive back pain; Gait difficulties; Psychiatric manifestations; Neurological examination at age 37 revealed difficulties in tandem gait, loss of balance segmental ataxia; Dysarthric speech; Dysphagia; Severe muscle atrophy; Unable to walk or stand; Respiratory insufficiency. Three additional sibs (P33a,b,c) showed an ataxic syndrome and psychiatric manifestations.

Myopatic Form					
34	F	5 yrs	Pt	Mus	Failure to thrive; Hypotonia; Similar twin-sister.
35	F	4 yrs	Pt	Mus	Feeding difficulties; Ventricular hypertrophy; Retin alterations; Low weight increasing; Loss of consciousness.
36	M	50 yrs	Pt	Mus	Intermittent postprandial vomiting; gastrointestinal dysmotility, episodes of diarrhe; constipation; Severe cachexia and numbness distally in the four limbs. Mild generalized weakness; Diffuse muscle atrophy.
37	F	5 yrs	Pt	Mus	Developmental delay; Hypotonia; Unable to walk and talk.
38	M	9 Mo	Pt	Mus	BTHS: Hyperlactacidemia; cardiomyopathy, skeletal myopathy, growth delay, neutropenia, and urinary excretion of 3-MGCA.
39	M	2 yrs	Nth	Fibr	Generalized epilepsy; ATP syntesis and fresh muscle oxidation rate decreased.
40†	F	1 Days	Pt	Mus	Cardiac failure; Congenital anemia; Cardiomegaly; Miocardic hypertrophy; Metabolic acidosis; Perinatal asphyxia; Died with 5h of life.
41	M	38 yrs	Nth	Fibr	Chronic fatigue; Generalized muscle weakness.
42†	F	10 Mo	Pt	Mus	Psychomotor delay; Hypotonia; Spasticity; Neurosensorial deafness; Epilepsy; Urinary excretion of 3-MGCA; Hyperlactacidemia; Nephew (P42a) deceased in the neonatal period with hypotonia, nystagmus, feeding difficulties and urinary excretion of 3-MGCA; Niece (P42b) with a similar clinical phenotype.
43	F	7 yrs	Pt	Mus	Hypotonia; Failure to thrive; Cardiomyopathy; Lipidic myopathy.
44	F	2 Mo	Pt	Mus	Hypotonia; Feeding difficulties; Weight loss; Brother deceased with a similar clinical phenotype.
45†	F	4 Days	Pt	Mus	Respiratory insufficiency; Metabolic acidosis; Multiple organ failure.
46	M	1 Mo	Pt	Mus	Hypotonia; Failure to thrive; Hypertelorism.
47	M	3 yrs	Pt	Mus	Feeding difficulties; Hypotonia; Failure to thrive.

Legend: M - Male; F - Female; Mo - Months ; yrs - Years; Pt - Portugal; Br - Brazil; It - Italy; Nth - The Netherlands; Mus - Muscle; Liv - Liver ; Bld - Blood ; 3-MGCA - 3-methylglutaconic acid; C4DC - succinylcarnitine; BTHS – Barth syndrome; MMA - methylmalonic acid.

3.2. Methods

3.2.1. DNA / RNA extraction and quantification

i) Total genomic DNA was extracted from different tissues, using commercial kits: the EZ1 DNA Blood 350 µl Kit (QIAGEN®) was used for blood samples, and Puregene Tissue kit (Gentra), for muscle and liver biopsies.
ii) Total RNA was extracted from different tissues, using commercial kits: PAXgene blood RNA kit (PreAnalitiX) was used for blood samples, and RNeasy Mini Kit (QIAGEN®), for skin biopsies.

NanoDrop® ND-1000 UV-Vis Spectrophotometer was used for nucleic acids quantification and purity.

3.2.2. Real-time qPCR

The comparison of techniques such as Southern-blot and real-time quantitative polymerase chain reaction (qPCR) allowed to conclude that qPCR is a more sensitive, faster and specific technique, and thus more appropriate for a better and accurate diagnosis of mtDNA depletion and multiple deletions (Chabi *et al.*, 2003; Bai and Wong 2005).
Calibration curves were prepared in triplicate for two mitochondrial genes (*ND1* and *ND4*) and one nuclear gene (*rRNA18S*), using serial dilutions of the PCR product of the target gene. The primers used for this assay are described in supplementary material.
MtDNA copy number (mtDNA depletion) was calculated from the standard curve and threshold cycle number using the ratios *ND1/18SrRNA* or *ND4/18SRNA*, and the *ND4*/*ND1* ratio was used to determine the presence of mtDNA deletions.
For qPCR reaction was used 1.25 µl of 1x GoTaq (Promega), 1.5 µl of each primer – forward and reverse (5 mM) (Supplementary material), 3 µl of $MgCl_2$ 25mM (Promega), 2 µl of dNTPs 2.5 mM (Promega), 1.25 µl of Eva Green (Biotium), 5 ng of DNA (sample or calibrator) and water in a final volume of 25 µl. The qPCR amplification conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15s of denaturation at 95°C and 60s of annealing/extension at 60°C. At the end of the amplification process, melting curves were analyzed between 60-95ºC (temperature transition of 0.1 ºC/s), with continuous fluorescence monitoring to control for the absence of nonspecific products. The fluorescent signal intensity of PCR products was recorded and analyzed in a Rotor-Gene 6000 (Corbett, Life Sciences).

The cycle threshold (Ct) value within the linear exponential phase was used to construct the standard curve. Using the comparative Ct method, outlined in reference (Livak and Schmittgen, 2001), the starting copy number of the unknown samples was determined in comparison with the known copy number of the calibrator sample, using the following formula: $\Delta\Delta Ct$ = [ΔCt 18S rRNA (calibrator sample) – ΔCt mtDNA (calibrator sample)] – [ΔCt 18S rRNA (unknown sample) – ΔCt mtDNA (unknown sample)]. The relative gene copy number was calculated by the expression $2^{-(\Delta\Delta Ct)}$. Values in patients were compared to results obtained in five normal individuals.

3.2.3. Mutational screening of genes associated with cross-talk disorders

Molecular screening for mutations in the genes associated with cross-talk disorders, such as *POLG*, *C10orf2* (*TWINKLE*), *DGUOK*, *MPV17*, *TK2*, *RRM2B*, *SUCLA2*, *SUCLG1* and *TYMP* was performed by PCR, followed by direct sequencing. Additionally, some other nuclear genes, recently described in the literature and associated with these diseases, were also screened: *RARS*, *TRMU*, *TSFM*, *TUFM*, *GFM1*, *TAZ* and *TTC19*.

PCR amplification

For PCR reactions a commercial mix was used: ImmoMix Red® (Bioline) containing IMMOLASE™ DNA polymerase, $(NH_4)_2SO_4$, Tween 20, $MgCl_2$, dNTPs and Tris-HCl buffer. Primers were designed using the program Primer 3.0 (http://frodo.wi.mit.edu/primer3/) in order to flank the coding sequences and exon-intron junctions of the genes described above (see supplementary material). All primers (Forward and Reverse) were tagged with M13 universal sequences and used at a 50 pmol/µl.

The program *Fast PCR Professional* 5.1 was also used to predict the formation of dimers and to evaluate the quality of the design primers (Kalendar *et al.*, 2009).

For each fragment the PCR conditions were optimized using annealing temperature gradients. The optimal annealing temperatures for each fragment are indicated in supplementary material. The hot start PCR protocol included an initial denaturation step at 95 ºC for 10min, followed by 35 cycles of amplification, which consists on a step of denaturation at 95 ºC – 1min; annealing at variable temperature, depending on the fragment – 1min and extension at 72 ºC – 1min. A final extension step was done at 72 ºC, lasting between 5min and 10min, depending on the size of the fragment amplified.

The PCR amplification product was analyzed in an 2 % Agarose (*Lonza-Seakem® LE Agarose*) with SYBR Green (*Roche*) in Tris-acetate-EDTA (TAE) buffer and visualized in a *Gel Doc System* (*Bio-Rad*).

Sequencing analysis

PCR amplified fragments were purified with ExoSAP (GE Healthcare) and sequenced with BigDye® Terminator v1.1 Cycle Sequencing Kits (Applied Biosystems) in an ABI 3130xl Genetic Analyzer (Applied Biosystems).

For amplicons purification 1 µl of Exo-SAP and 2 µl of PCR product were incubated at 37ºC for 30 min followed by a step at 80ºC for 15 min for enzyme inactivation.

For sequencing the following program was used: initial denaturation step at 94ºC for 4min, followed by 25 cycles consisting in 94ºC for 10sec, 50ºC for 6sec and 72ºC for 4min; and a final step of elongation at 72ºC for 5min. The *DyeEx 96 Kit* (*QIAGEN*) was used to remove the unincorporated dye terminators, following the instructions of the manufacturer.

Mutations were confirmed by resequencing a second amplicon with both sense and antisense primers. Segregation of the mutations in available family members and frequency of novel mutations in a panel of 200 ethnically-matched healthy chromosomes was performed by direct sequencing.

Bioinformatic analysis tools

The sequences obtained were compared to the reference of the respective gene using the Ensemble Genome Browser (http://www.ensemble.org), and the variants found were searched for in HGMD® Professional database (https://portal.biobase-international.com/hgmd/pro/start.php?).

For novel variants Clustal W program (http://www.ebi.ac.uk/Tools/msa/clustalw2/), multialignments were done to determine the degree of evolutionary conservation of the amino acid residue involved (Thompson *et al.*, 1994). Furthermore, the amino acid substitution was analysed by PolyPhen (PolyPhen: Prediction of functional effect of human nsSNPs. http://genetics.bwh.harvard.edu/pph/), to predict if an amino acid change is likely to be deleterious to protein function. Profile scores of >2.0 indicate that the polymorphism is probably damaging to protein function whereas scores of 1.5–2.0 are possibly damaging, and scores of <1.5 are indicative of a benign variant. To confirm results from PolyPhen, we also used (i) the MutPred Server (http://mutpred.mutdb.org/), which generates a probability for a given mutation to be deleterious to protein function (scores that range from 0 to 1), and the likelihood of it being pathogenic (scores over 0.5) and (ii) SIFT (http://sift.jcvi.org/), which yields a score of ≤0.05 if the amino acid substitution is predicted to be damaging and >0.05 if it is predicted to be tolerated.

3.2.4. BN-PAGE

For BN-PAGE, mitochondria-enriched fractions were isolated from cultured fibroblasts (Nijtmans *et al.*, 2002).

The harvested cells were treated with digitonin, which selectively dissolves membranes containing cholesterol, saving the IMM. The undissolved IMM fraction was then collected by centrifugation and the membrane proteins were solubilized with dodecyl-β-D-maltoside. Briefly, the phosphate-buffered saline (PBS) washed cells were resuspended in approximately 100 µl of PBS. The same volume (100 µl) of cold digitonin was added (C=8 mg/ml in PBS) to the cell suspension. This mixture was maintained in ice for 10min, after which 1 ml of cold PBS was added. It was then centrifuged for 5min, at 10000g, at 4ºC. The supernatant was discarded and the pellet washed once more in 1 ml of cold PBS. The pellet obtained can be frozen or immediately dissolved.

In order to isolate mitochondria, the cell pellet was washed twice in the homogenization buffer (0.25 M sucrose, 1 mM EGTA, 10 mM HEPES/NaOH, 0.5% bovine serum albumin, (pH 7.4), being centrifuged at 1500g for 5min. The pellet was resuspended in approximately 5 mL of the homogenization buffer and kept in ice. It was added 10 µl of Protease (100 µg/ml), allowing the disruption of the membrane and after 7min, a mixture containing 5 ml of homogenization buffer with 5 µl of pepstatin (1 mg/ml), 5 µl of leupeptin (1 mg/ml), and 10 µl of PMSF (1 M) was added. This suspension was homogenized with 12 up and down strokes in a rotating glass Teflon homogenizer and then centrifuged at 1500g for 8min at 4℃. The supernatant was transferred to another tube and was kept in ice. The pellet was dissolved, homogenized and centrifuged as above. The supernatant obtained was added to the first one, and a crude mitochondrial pellet was collected by centrifugation at 14600g for 12min at 4℃. This pellet was resuspended in 140-200 µl of washing buffer (10 mM Tris/HCl, 1 mM EDTA, 0.25 M sucrose, pH 7.5), which was frozen or immediately suspended (Garcia *et al.,* 2000).

After measuring the protein, using the bicinchoninic acid (BCA) protein assay, at λ=562 nm, lysis of mitochondria-enriched fractions for BN-PAGE, from fibroblasts was obtained. Once solubilized, the pellets (150-250 µg of protein) were vigorously pipetted in 100 µl buffer containing 1.5 M aminocaproic acid, 50 mM Bis–Tris/HCl, pH 7.0 with 1% (v/v) of lauryl maltoside (Sigma Aldrich), and incubated on ice for 30min. They were centrifuged at 20000g for 30min at 4ºC. The supernatant was transferred to a new Eppendorf tube, after which a new protein determination was done. The amount of protein needed was transferred to a new eppendorf tube and 10% of sample buffer (750 mM aminocaproic acid, 50 mM Bis–Tris/HCl, pH 7.0, 0.5 mM EDTA, and 5% Serva Blue G) was added. This sample is ready to load on a gel, or stored for at least a month at -80 ºC.

The assembly and level of expression of complex III in the patient fibroblasts was analyzed using BN-PAGE and second-dimension (2D) SDS-PAGE (Schagger and von Jagow 1991). Detergent-solubilized IMM enzyme complexes (30-60 µg proteins) were separated by BN-PAGE in a linear 5-13% acrylamide gradient gel (Nijtmans *et al.*, 2002; Calvaruso *et al.*, 2008).

The gradient gel was polymerized in a gel caster, using 1.5 mm thick spacers. Two mixtures were prepared, one corresponding to the 5% gel and other to the 13% gel (see supplementary material), and were added to the gradient maker. After polymerization of the previous gel, the stacking gel was also casted.

The electrophoresis apparatus is set up, with the cathode buffer A (50 mM Tricine, 15 mM Bis-Tris, 0.02% Serva Blue G, pH 7.0) in the inner compartment and the anode buffer (50 mM Bis-Tris pH 7.0) in the outer compartment. The samples were loaded and electrophoresed at 4ºC, until they have entered the stacking gel, at 30 V for 30min, after which the voltage was increased to 80 V. When the front has reached the middle of the separating gel, the cathode buffer A was replaced for the cathode buffer B (50 mM Tricine, 15 mM Bis-Tris, pH 7.0). Electrophoresis continued at 80 V until the blue dye front reached the end of the gel.

After that, proteins were either subjected to western-blot or the lanes were cut off and the enzyme complexes were separated into subunits by 2D SDS-PAGE.

3.2.5. 2D-SDS-PAGE

For further separation in a second-dimension SDS-PAGE, the lanes from first-dimension BN-PAGE were cut out. Gel strips were equilibrated for 1h in a dissociating solution (1% SDS and 1% β-mercapthoethanol) and excess of solution was drained with filter paper. The gel strip was placed into the top of the glass plate and a second-dimension SDS-PAGE of the same thickness was done. A separating gel of 12% was poured and overlaid with water. After polymerization, we added the 5% stacking gel , including strips around the gel. The electrophoresis apparatus was prepared with the cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.2) and the anode buffer (0.2 M Tris-HCl, pH 8.9), and started at 30 V for 30min and continued at 80 V (2-4h) or at 20 V, overnight. Immunoblotting were performed according to standard protocols (described above).

3.2.6. Western-blot

Proteins separated either by BN- or SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane (*Immobilion-P transfer membrane, Millipore*) and subjected to Western blotting according to standard protocols (Towbin *et al.*, 1979). First the PVDF membranes were cut in the proper size and immersed in methanol for about 30s, which was then discarded and substituted by the transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol for SDS-PAGE membranes and 25 mM Tris, 192 mM Glycine, 20% Methanol and 0.02% SDS for BN-PAGE membranes). The gel was removed from its cast and immersed in the transfer buffer. The sponges, filter papers had been previously wetted in the same buffer. The sandwich was assembled in the following order from cathode to anode, avoiding air bubbles: sponge – filter paper Whatman 3MM – gel – PVDF membrane – filter paper Whatman 3MM – sponge. The sandwich was placed inside the transfer apparatus and filled with cold transfer buffer, which was then set up at 4 ºC on a stir plate. The transfer was done at 100 V for 1h.

When finished, depending if the membranes were from BN-PAGE or SDS-PAGE, they were treated in different ways. The membrane from SDS-PAGE was stained with Ponceau (Ponceau S 0.3%, TCA 3%) and washed two times with water. Instead, the PVDF membrane from BN-PAGE was air dry for at least 1h, after which was washed quickly two times with methanol and once with water.

The membranes were then blocked in milk 5%, prepared in 0.1% Tween 20 – Tris-Buffered Saline (T-TBS) (20 mM Tris-HCl, 137 mM NaCl, 1% Tween 20, pH 7.6) one hour at room temperature or overnight at 4ºC.

After the blocking, membranes were successively incubated with primary antibodies diluted in 2.5% milk/T-TBS (Supplementary material), for 60min at room temp, or overnight at 4 ºC. Different monoclonal antibodies against the human OXPHOS subunits were used (Table 3), all of which purchased from Mitosciences (Eugene, OR, USA). The membranes were then washed three times, for 5min, with T-TBS and incubated with the secondary antibody (diluted in 2.5% milk/T-TBS – 1:50000) for 1h at room temperature. The *Peroxidase-conjugated anti–mouse IgGs* (*GE Healthcare)* was used as the secondary antibody. Prior to the signal detection, the membranes were washed two times with T-TBS for 5min and one time with TBS (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) for 10min. The signal was detected using the *Immobilon Western Chemiluminescent HRP Substrate detection kit (Millipore Corporation*) and the fluorescence were quantified using the software *Quantity One (Bio-Rad)*.

Table 3 – Monoclonal antibodies against the human OXPHOS subunits used in this study.

Monoclonal Antibody	Final Concentration
CI subunit NDUFA9	1 µg/ml
CII subunit 70	0,1 µg/ml
CIII subunit Core 2	1 µg/ml
CIV subunit COX II	1 µg/ml
CV subunit alfa	1 µg/ml
Porin	0,1 µg/ml

3.2.7. Multiplex ligation-dependent probe amplification - MLPA

Multiplex Ligation-dependent Probe Amplification (MLPA®) is designed to detect deletions/duplications of one or more sequences in the aforementioned gene(s) in a DNA sample. In this work two commercially available probe sets were used: SALSA MLPA KIT P089-A1 TK2 and SALSA MLPA probemix P010-A2 POLG (both from MRC-Holland), for detecting copy-mumber variation in *TK2*, *DGUOK* and *C10orf2*, and in the *POLG* gene, respectively.

This is a high-throughput method developed to determine the copy number of up to 50 genomic DNA sequences in a single multiplex PCR-based reaction. The MLPA reaction results in a mixture of amplification fragments between 100 to 500 nt in length which can be separated and quantified by capillary electrophoresis. In contrast to a standard multiplex PCR, only one pair of PCR primers is used in the MLPA PCR reaction (Figure 16), resulting in a more robust system.

1) DNA denaturation					
	1.	98 °C	*5 minutes*		
	2.	25 °C	*pause*		
2) Hybridisation reaction					
	3.	95 °C	*1 minute*		
	4.	60 °C	*pause*		
3) Ligation reaction					
	5.	54 °C	*pause*		
	6.	54 °C	*15 minutes*		
	7.	98 °C	*5 minutes*		
	8.	15 °C	*pause*		
4) PCR reaction					
	9.	60 °C	*pause*		
		35 cycles:	•	95 °C	*30 seconds*
			•	60 °C	*30 seconds*
			•	72 °C	*60 seconds*
	10.	72 °C	*20 minutes*		
	11.	15 °C	*pause*		

Figure 16 - PCR program for the MLPA reaction.

Each MLPA probe consists of two oligonucleotides which have to hybridise to adjacent DNA targets in order to be ligated. Since only ligated probes can be amplified exponentially during the subsequent PCR, the amplification of a given probe depends on the presence of its target DNA. Moreover, the relative amount of each probe's amplification product is a measure for the relative quantity of that probe's target sequence in the DNA sample. To quantify this relation, two calculations need to be done: (1) establishing the relative signal of each probe as compared to that of the other probes (intra-sample normalisation); (2) establishing the relative signals in the sample under study compared to those of reference samples (inter-sample normalisation). Upon normalisation, it is possible to establish whether any deletions or duplications in the target DNA have occurred. Heterozygous deletions of recognition sequences should give a 35-50% reduced relative peak area of the amplification product of that probe.

3.2.8. Measuring ROS and ATP production in cultured primary cells

Human fibroblasts were obtained from skin biopsies and grown in regular Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum, 4.5 g/L glucose and 50 µg/ml uridine or in glucose-free medium supplemented with 5 mM galactose, to induce a stress culture condition.

The cells were harvested with trypsin and washed two times with PBS. Depending on the application, the pellet was immediately used or frozen at -80 ºC.

ROS production

Fibroblasts cells at a density of 1×10^4, incubated in a 96-well plate, were analyzed 72h post-treatment with the following compounds: 0.5 mM AICAR (Tocris, Bioscience, Bristol, UK), 200 µM bezafibrate (Sigma-Aldrich) and 3,5 µM riboflavin (Sigma-Aldrich), for rate of ROS. After washing with respiration buffer (142 mM NaCl, 10 mM HEPES, 2 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$) (Sigma, St. Louis, MO), supplemented with 1% FBS (ROS buffer), 100 µl of 5 µM 5-(and-6)-chloromethyl-2,7-dichlorodihydro-flouorescein diacetate, acetyl ester (CM-H2DCFDA) (Molecular Probes, Invitrogen, Paisley, UK) in ROS buffer were added to each well and incubated 15 min at 37°C.

After washing with the same buffer, the cells were incubated for 30 min at 37°C with 100 µl of ROS buffer with or without 100 µl H_2O_2 (500µM in buffer ROS) (Sigma-Aldrich). Wells were subsequently washed with the same buffer and after added another 100 µl of ROS buffer fluorescence could be detected. Fluorescence was measured at excitation and emission wavelengths of 490 nm and 527 nm, respectively, using a Varian Cary Eclipse Fluorescence plate reader (Agilent Technologies).

ATP production

ATP levels were assayed using the Luminescence ATP Detection Assay System (PerkinElmer Life Sciences). Briefly, cells were seeded in a 96-well plate (1 x 10^4 cells/well) incubated in DMEM for 48 h. After discarding the medium, wells were washed with ATP record buffer (156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 20mM HEPES, pH 7.35) and incubated for 2 h in ATP record buffer with the following conditions: 10 mM glucose; 10 mM glucose plus 2.5 μg/ml oligomycin (glycolytic ATP generation) and 5 mM 2-deoxy-D-glucose plus 5 mM pyruvate (oxidative ATP production). Cells were incubated with the luciferin/luciferase reagent and ATP production in samples was measured using an Orion L microplate luminometer (Berthold Detection Systems GmbH).

CHAPTER IV

RESULTS

RESULTS

The population presented in this study was selected from the cases referred to the Laboratory of Mitochondrial Pathology and with a clinical or biochemical suspect of disorders of intergenomic communication. Eligible patients were recruited if they satisfied clinical, biochemical and/or morphological criteria for disorders of intergenomic communication. Figure 17 shows a scheme recapitulating the approach used to select patients.

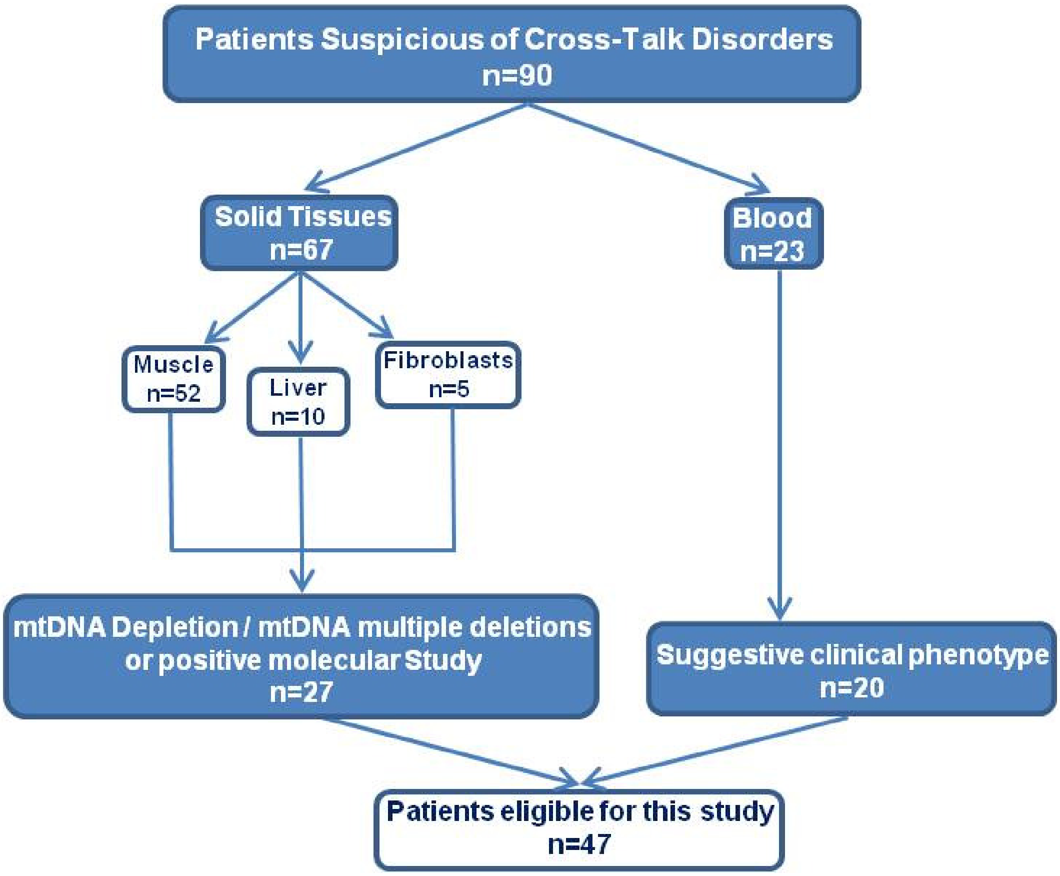


Figure 17 - Scheme illustrating the patients eligible for this study.

In a total of 90 cases, we analyzed the relative mtDNA/nDNA ratio by real-time qPCR in 67 samples obtained from solids tissues and identified a significantly low level of mtDNA (<30% range of control samples) in 17 patients (defined as “primary” mtDNA depletion). In an additional set of 8 patients, we detected a moderate reduction (40-60% of the control range) (“secondary” depletion). In one patient we did not detect mtDNA depletion or multiple deletions, yet he was considered eligible for this study, based on his suggestive clinical features. A patient with multiple deletions was also identified by real-time qPCR, through the ratio *ND4*/*ND1*. In addition, 20 of the 23 patients in whom we could obtain DNA only from blood were also analyzed in this study based on their suggestive clinical features.

The remaining patients were not included in this study because either they did not show defects of intergenomic communication or they had different metabolic diagnosis.

4.1. "Primary" mtDNA depletion

A primary mtDNA depletion was detected in 17 patients with a severe to moderate mtDNA depletion or clinical phenotype suspicious of MDS. A subset of these patients (Figure 18) presented mutations in the following genes associated with MDS: *MPV17*, *SUCLA2*, *DGUOK, C10orf2 and POLG,* whereas one patient presented mutations in the *TAZ* gene. In this group we included two patients expected to have "primary" mtDNA depletion, however in P18, with a pathogenic *POLG* mutation commonly associated with AHS no mtDNA depletion was found in the studied tissues. Additionaly in P27 with a pathogenic SUCLA2 mutation, we expected a more pronounced mtDNA reduction, but the tested tissue (fibroblasts) was not adequate to illustrate such mtDNA content reduction, as it would be expected in muscle.

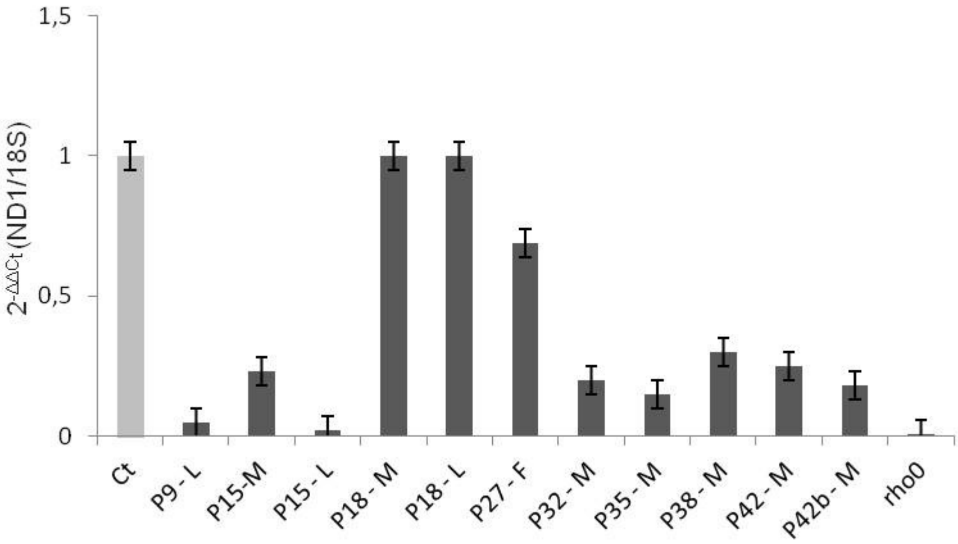


Figure 18 – Bar chart showing the relative quantification of mtDNA content by qPCR using the $2^{-\Delta\Delta Ct}$ method. The error bar in controls (Ct) indicates three times the standard deviation. Data represents the mean ± SD of three different determinations; rho0 are cells experimentally deprived of their own mtDNA. L – Liver; M- Muscle; F – Fibroblasts.

MPV17

The hepatocerebral forms of mtDNA depletion syndromes typically manifest soon after birth and with premature death in childhood (Suomalainen *et al.*, 2010). Presentation is usually with early liver failure, followed by development delay and muscle weakness during the first year of life, as well as lactic acidosis and hypoglycemia (El-Hattab and Scaglia 2013). These conditions are associated with mutations in at least five genes (Rötig *et al.*, 2009) though further heterogeneity is expected. An ample array of *MPV17* mutations (Figure 19) has been described in about 30 patients from different ethnicities.

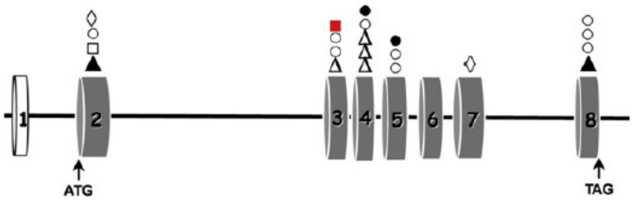


Figure 19 - Described mutations in the *MPV17* gene. ○Missense mutation; ●Nonsensemutation; Δ Small deletion; ▲ Gross deletion; ◊ Small insertion; □ Splicing mutation; ■ **c.186+2T>C**.

We identified a Brazilian infant (P15), the second child of apparently unrelated healthy parents, who presented neonatal hypoglycemia, digestive bleeding and sepsis in the first week of life was included in this study. Laboratory tests also showed elevated levels of total and free serum bilirubin and transaminases. Amino acids were normal whereas organic acids revealed the presence of lactic acid (3X normal values) and TCA cycle intermediates at age 2 months. Shortly after the patient manifested failure to thrive, severe hypotonia, nystagmus, poor echoarchitecture at liver ultrasound, and cirrhotic ascites. He died at 5-months with hepatic insufficiency associated with bleeding and multiple organ failure. The child had a severe mtDNA depletion (residual mtDNA levels were 2% in liver and 23% in muscle) and molecular studies detected a homozygous c.186+2T>C replacement in *MPV17* (Figure 20), a variant already reported with poor prognosis in a singleton (El-Hattab *et al.*, 2010). The c.186+2T>C was also detected in the proband's elder sister (P15a) who had presented at birth with neonatal hypoglycemia, failure to thrive and hepatic insufficiency, later developed seizures and hypotonia, and died at 10 months. The mutation was heterozygous in the parents who were from nearby villages in south Brazil. This study expands the ethnic background of *MPV17*-mutated patients and reinforces the lethal nature of the c.186+2T>C probably through skipping of critical protein domains.

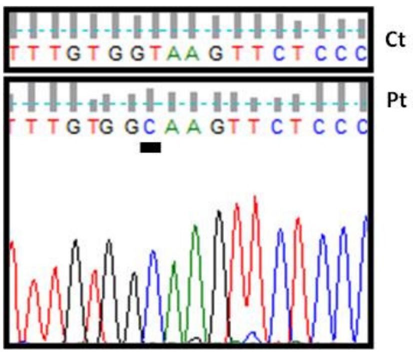


Figure 20 - Electropherogram of the *MPV17* gene flanking the homozygous splice site mutation (c.186+2T>C). The mutated base identified in the patient (Pt) is underlined. Wild-type sequence in a control (Ct) is also shown.

DGUOK

Deoxyguanosine kinase (dGK) deficiency is a frequent cause of the hepatocerebral form of MDS, Approximately 100 individuals have been reported with this form which can present as a multi-organ failure in neonates and as isolated hepatic disease later in infancy or childhood (El-Hattab and Scaglia 2013). More recently, *DGUOK* mutations have been reported in one patient with clinical and autopsy findings consistent with neonatal hemochromatosis and mtDNA depletion, and in individuals with adult-onset mitochondrial myopathy and mtDNA multiple deletions in skeletal muscle (El-Hattab and Scaglia 2013). Hepatic dysfunction is progressive in most individuals regardless of the presentation and is the most common cause of death.

Neonatal hemochromatosis was suspected initially in one of the studied patients (P11). He was the third child of consanguineous parents, with a brother deceased (P11a) during neonatal period with the diagnosis of haemochromatosis. Since the first day of life, he presented with generalised hypotonia, feeding difficulties with poor suction reflexes and hypoglycaemia. Wide anterior fontanel, hypotelorism and macroglossia were noticed. At third day of life, acute liver failure emerged. Neonatal haemochromatosis was considered and he was transferred to a center hospital for liver transplantation. He had hyperlactacidemia, high lactate/pyruvate ratio, high ferritin level, elevated α-fetoprotein level and elevated tyrosine plasma concentration, with absence of succinylacetone in urine. After a period of stabilisation, cholestatic liver dysfunction with frequent hypoglycaemia episodes and neurologic dysfunction with rotary nystagmus manifested at 3 months of age. A severe reduction of mtDNA content was expected to occur in liver biopsy (not available). We detected a *DGUOK* homozygous missense mutation (p.L250S; c.749T>C) already described by Villarroya and collaborators in 2009. The mutation, was identified in blood DNA from this patient (Figure 21). This mutation was also presented in his brother who had died earlier with a possible diagnosis of hemocromatosis. The index case was submitted to liver transplantation at 5 months of age. Liver morphology showed cholestasis and cirrhosis. One year later, graft function was normal (with minimal immunosuppression), however nystagmus persisted and he has visual impairment, severe psychomotor retardation and failure to thrive.

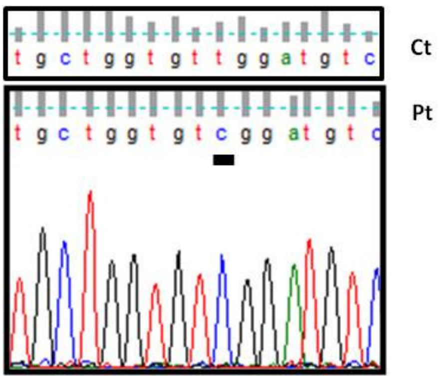


Figure 21 - Electropherogram of the *DGUOK* gene flanking the homozygous mutation p.L250S (c.749T>C). The mutated base identified in the patient (Pt) is underlined. Wild-type sequence in a control (Ct) is also shown.

SUCLA2

Succinyl-CoA synthase is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinate and ATP from succinyl-CoA and ADP in the TCA. This enzyme is made up of two subunits, α and β, encoded by *SUCLG1* and *SUCLA2*, respectively.

We identified a 17-month-old-boy (P27), the first child born to unrelated parents, who presented since birth severe muscular hypotonia and frequent vomiting. Growth retardation and developmental delay were noticed since the age of 3 months. Initial investigations disclosed a male karyotype with normal thyroid, renal and hepatic functions, as well as normal blood lactate levels. Plasma amino acids and urinary organic acid profiles showed unspecific abnormalities. A brain MRI disclosed slight cerebral atrophy and normal myelin pattern (Figure 22); a cardiac ultrasound was normal.

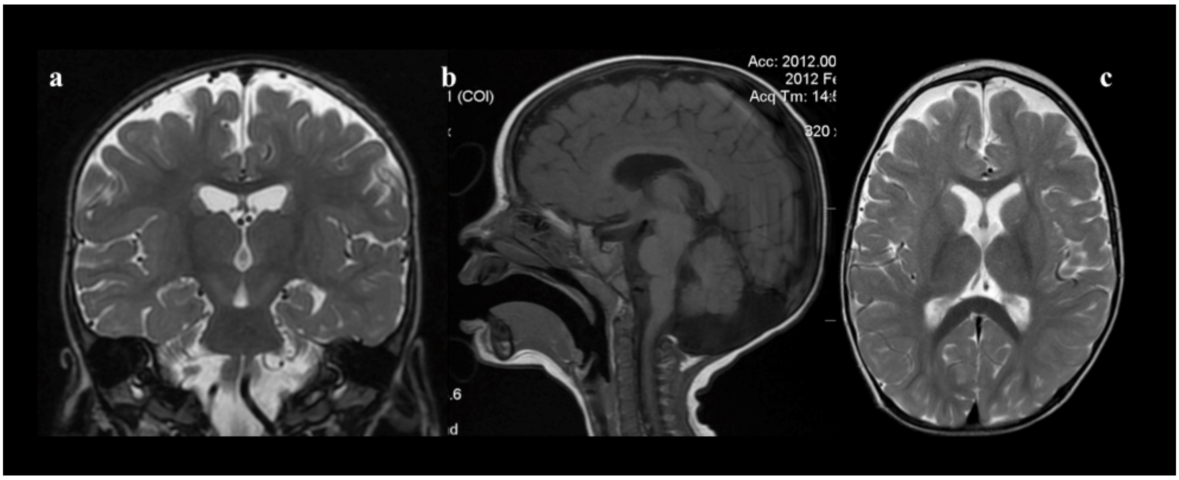


Figure 22 - Brain MRI scan of the patient. Imaging disclosed mild cerebral atrophy and a slight hyopointensity of basal ganglia. Coronal **(a)**, sagittal **(b)** and axial **(c)** scans are shown.

The baby boy was referred to a Central Hospital at age 16.5 months because of a possible inherited disorder of metabolism. At that age, his physical examination was significant for developmental delay, failure-to-thrive, microcephaly, muscular atrophy with dystonic posturing, recurrent vomiting and high ammonia level (115 μM, normal: 25-44 μM) in blood. During hospitalization, metabolic workup revealed a compensated metabolic acidosis with persistently high serum lactate (2.2 to 9 mM, normal <1.3), and lactate/pyruvate ratio. There were also fluctuating hyperammonemia (up to 75 μM), high alanine, proline and glycine levels in plasmatic aminoacid profile, “mildly” elevated MMA and significantly high urinary levels of TCA cycle intermediates (methylcitrate, lactate, carnitine esters and 3-hydroxyisovaleric acid). Acylcarnitines profile showed an elevated succinylcarnitine. Brainstem auditory evoked potentials showed a pattern suggestive of moderate sensorineural hearing loss. Based on laboratory findings, the patient was put on a protein restricted diet, with nasogastric tube feeding and vitamins and coenzyme supplementation. At the age of 18 months, the child manifested axial hypotonia (no head control), severe generalized dystonia, muscle wasting and constipation. All anthropometric parameters were below the 5th centile (weight 7.1 kg, height 76.8 cm, head circumference 45.2 cm) in spite of adequate caloric intake. At age 24 months, his global developmental quotient (DQ) was below 50 (< 1 percentile), with a mental age of 6 months, using the Griffths scale (Griffiths, 1984). Brain MRI scans showed mild atrophy and abnormal signals in deep brain at that age. Since parents refused to grant permission for a diagnostic muscle biopsy, the child underwent a punch skin biopsy with written parental consent.

Direct sequencing of *SUCLA2* in this patient revealed a homozygous mutation, p.M329V (c.985A>G), in exon 8 (Figure 23A). The parents presented a cognitive impairment and were heterozygous carriers of the mutation. The missense change was novel and not detected in a large set of in-house ethnically-matched control chromosomes and in dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/) and Exome Variant database (evs.gs.washington.edu/EVS/).

The mutation seems predictably deleterious after assessments in silico with Polyphen2 (genetics.bwh.harvard.edu/pph2/index.shtml), ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and SIFT (sift.jcvi.org/), and affects an amino acid that is highly conserved in different species through evolution (Figure 23B; 23C; 23D).

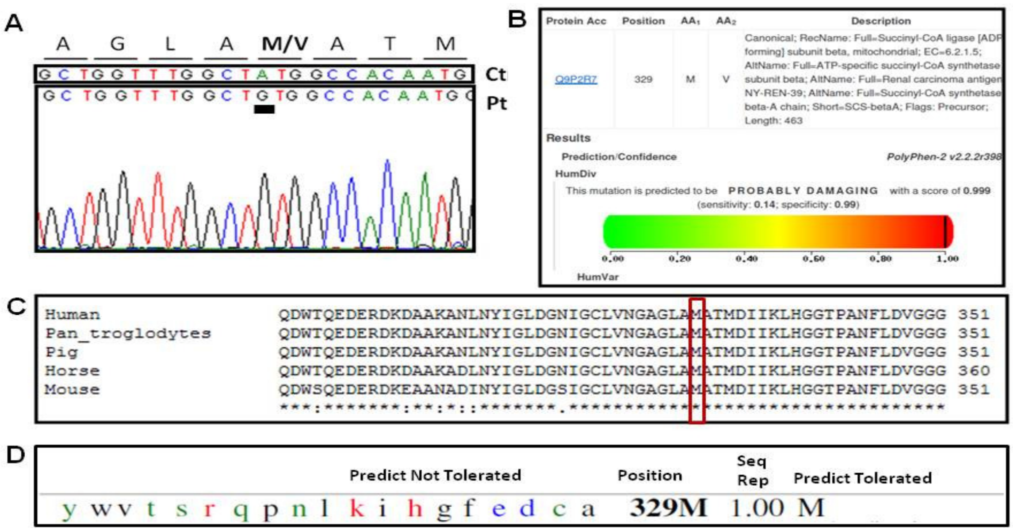


Figure 23 - Molecular analyses of a patient harboring a novel mutation in *SUCLA2*. A) Electropherograms of the *SUCLA2* gene flanking the homozygous p.M329V (c.985A>G) in the index case (Pt). The wild-type sequence in a control (Ct) is also shown. The mutated base is underlined; **B)** PolyPhen-2 prediction of functional effects of human variations; **C)** ClustalW aligments; **D)** Sift predictions.

A moderate reduction of mtDNA copy number (residual mtDNA levels were 69% of normal age matched controls) was observed in cultured skin fibroblasts after replacing galactose for glucose in the cell medium. Western blotting showed a ~30% decrease of SUCLA2 protein in patient's fibroblasts (Figure 24) with normally expressed components of the MRC complexes.

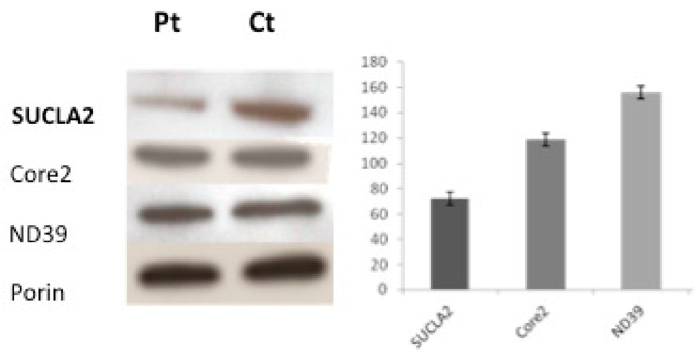


Figure 24 - Representative immunoblot analysis in fibroblasts homogenates from a control (Ct) and patient (Pt). Specific antibodies against SUCLA2 and subunits of complex III (Core 2) and complex I (ND39) were used. VDAC/porin was used to control for equal loading. The histogram shows the residual activity in the patient as compared to controls (n= 5). Data represents the mean ± SD of three different determinations.

Total and mitochondrial ATP production in skin fibroblasts were 22% and 50% ($p<0.03$), respectively when compared to normal control values (Figure 25A). We also observed a statistically significant increase of ROS production in basal condition and after short-term H_2O_2 treatment (Figure 25B, $p<0.05$). ROS production was then restored to basal levels upon treatment with 0.5 mM AICAR, a compound known to enhance cellular oxidative metabolism (Golubitzky *et al.*, 2011). Bezabibrate and riboflavin were also tested, however no improvements were observed (data not shown).

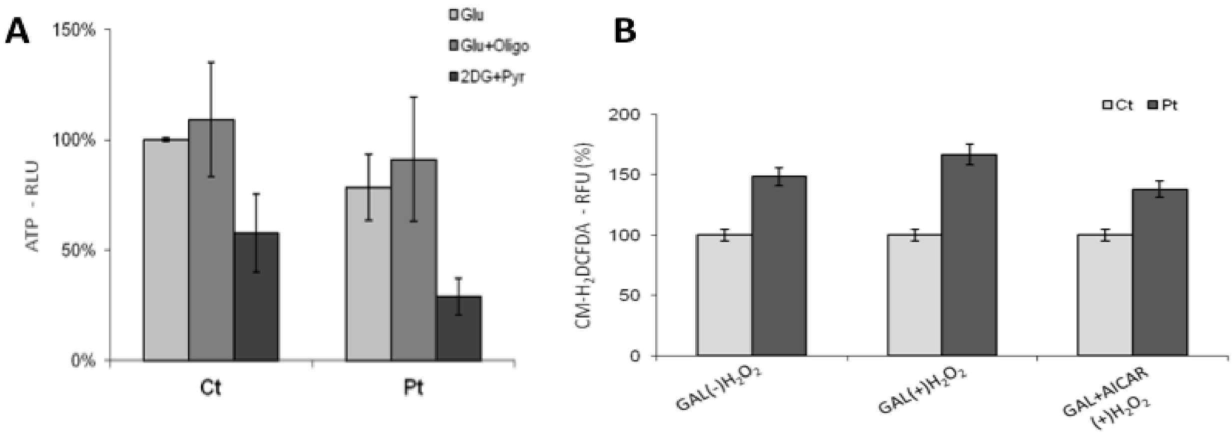


Figure 25 - Biochemical analyses in cells harboring the new c.985A>G/p.M329V in *SUCLA2*. A) Luminometric measurement of ATP expressed as relative luminescence units (RLU), in skin fibroblasts from controls (Ct) and patient (Pt) cultured in regular medium (RM) for 48 h. Cells were incubated 2 h in ATP record buffer supplemented with 10mM glucose (Glu), 2.5 µg/ml oligomycin (Oligo) and 5 mM 2-deoxy-Dglucose plus 5 mM pyruvate (2GD+Pyr). Data represents the mean ± SD of three different determinations. **B)** Reactive oxygen species (ROS) production measured by CM-H_2DCFDA and expressed as % of relative fluorescent units (RFU) in skin fibroblasts from controls (Ct) and the patient (Pt). Cells were cultured in medium supplemented with galactose (GAL) or Gal+AICAR 0.5mM, and incubated with or without 0.5mM H_2O_2 for 30 min. Data are mean ± SD of three different determinations. Significance (*) was set at $p<0.05$.

C10orf2

Although the spectrum of diseases attributable to *C10orf2* gene defects involves different clinical presentations, including infantile-onset spinocerebellar ataxia (IOSCA), adPEO and hepatocerebral MDS, the signs in IOSCA demonstrate a fairly distinct pattern. Among these, peripheral neuropathy seems to be the most common presenting feature in *C10orf2* defects, especially in infantile onset spinocerebellar ataxia and hepatocerebral syndrome but rarely seen in adPEO forms (Dundar *et al.*, 2012).

Detailed clinical and laboratory evaluations in one of the studied patients (P32), born to unrelated healthy parents, showed hypotonia and Pierre-Robin sequence (OMIM #311895) with mandibular retrognathism, partial syndactyly of 2nd-3rd fingers, bilateral strabismus, and eyelid asymmetry. The child manifested severe intellectual and developmental disability and nystagmus in the following months. Brain MRI revealed brain atrophy and a thin corpus callosum (Figure 26).

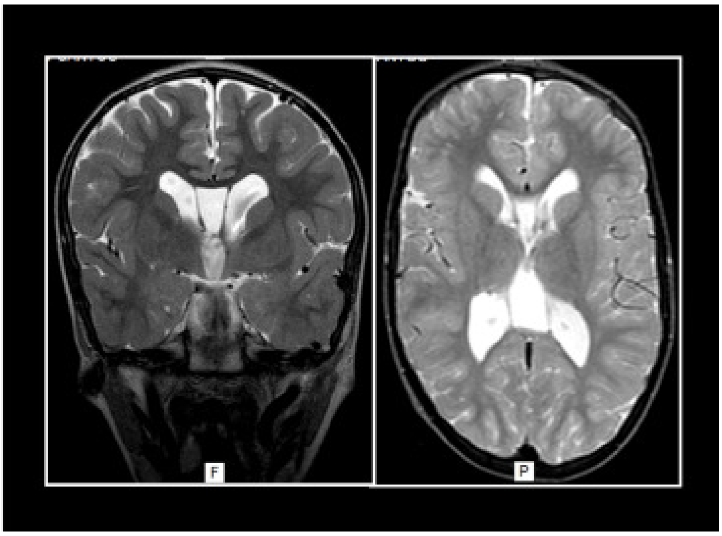


Figure 26 - Brain MRI (coronal and axial images). There was decreased intensity in the posterior periventricular white matter and a thinner corpus callosum. No pathological signal changes were seen in the striatum, brainstem and dentate nuclei. F-frontal; P-posterior.

Serum lactate levels were increased, OXPHOS activities in a skeletal muscle biopsy were in the low normal range and there was a severe mtDNA depletion in muscle (residual mtDNA levels were 20% of normal age matched controls).

In the patient we identified two “in cis” homozygous splice site mutations in *C10orf2* ([c.1593-3T>C + c.1593-5C>T]; Figure 27) predicted *in silico* to activate a new cryptic acceptor site and lead to missplicing. The novel mutations were observed in heterozygosity in the mother and in the healthy brother whereas the father was a non-carrier.

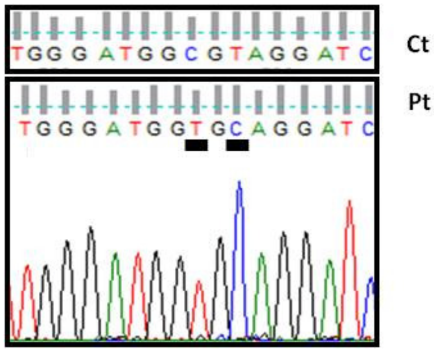


Figure 27 - Electropherograms of the *C10orf2* gene flanking the homozygous splice site mutations ([c.1593-3T>C + c.1593-5C>T]. The mutated bases identified in the patient (Pt) are underlined. Wild-type sequence in a control (Ct) is also shown.

Once we had ruled out false paternity and a multi-exon deletion on the paternal allele by MLPA (Figure 28), we genotyped three microsatellite markers and 12 SNPs flanking C10orf2 on chromosome 10q24. Whilst a number of markers were uninformative, SNP genotyping was also consistent with segmental uniparental dissomy (UPD) of the maternal chromosome 10 (Figure 29).

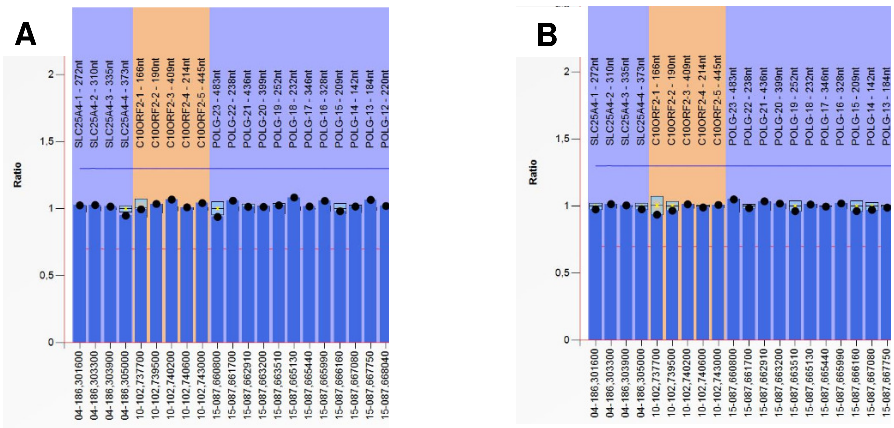


Figure 28 - MLPA analyses of *C10orf2 gene* (SALSA MLPA probemix P010-A2 POLG (both from MRC-Holland). Coffalyser Sample Plate Generator (S.P.G.) was used to automatically create sample plate files for MLPA analysis. A) Patient 32; B) Normal control.

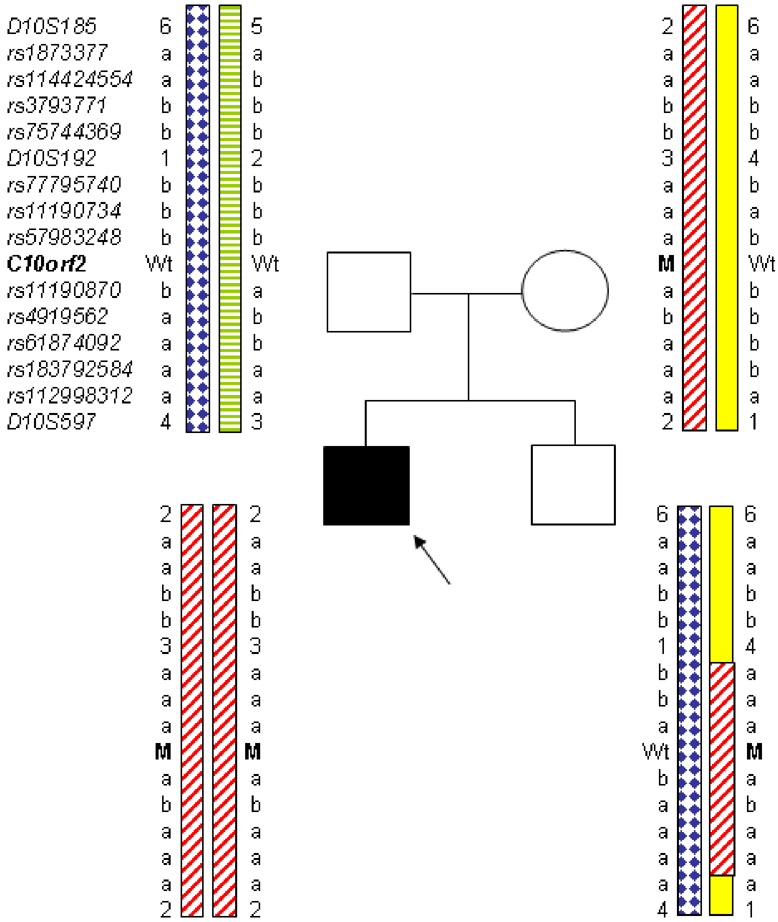


Figure 29 - Segregation analysis of polymorphic microsatellite markers and single nucleotide polymorphisms flanking the *C10orf2* gene suggested maternal UDP. Family tree: square, men; circle, women; filled symbol is the affected patient (arrow). M, *C10orf2* mutated sequences; Wt, wild-type sequence.

A similar condition has already been reported for *TYMP* and *DGUOK* (Douglas *et al.*, 2011; Haudry *et al.*, 2012) two other genes associated with relatively common forms of MDS.

Description of UDP in a patient presenting mutations in *TWINKLE* corroborates the need of a full molecular examination of apparently homozygous changes in MDS to define more precisely the risk of recurrence in subsequent pregnancies.

POLG

Diseases resulting from mutations in *POLG* are associated with an extremely heterogeneous spectrum of clinical presentations, ranging from adPEO and arPEO forms, to SCAE and AHS. Severe heterozygous mutations of *POLG*, often affecting the spacer (DNA-binding) and the catalytic polymerase domains of the enzyme, cause mtDNA depletion, and manifest in infancy or early childhood with severe liver and brain disease.

We identified four patients with *POLG* mutations:

P9, a boy with a mild global developmental delay at age 9 months, developed muscular hypotonia was noticed at age 15 months before the sudden onset of *status epilepticus.* In the 2 weeks prior to the admission he had had evidence of an eye deviation and he had been admitted to hospital a week before with a "viral" infection, when he had appeared floppy and had been vomiting. CSF oligoclonal bands and other routine tests were normal on that admission. The first seizure at the age of 18 months was followed by persistent seizures after which he remained markedly encephalopathic, having multiple seizures that were refractory to all antiepileptic drugs including sodium valproate. The preliminary brain MRI was normal, but then basal ganglia changes and some atrophy were found. A further scan 3 months later showed a new lesion in the right parietal cortex. Brain MRI spectroscopy showed an increased choline peak in the right frontal cortex and increased lactate peak in the posterior fossa. The patient then developed mildly abnormal liver function suggesting AHS but assessment of OXPHOS complex activities was not possible in a liver biopsy. Subsequent EEG recordings revealed a very disordered background with epileptiformic discharges. The patient died due to intractable epilepsy and liver failure at age 2 years. The parents refused a post mortem examination. Determination of mtDNA/nDNA ratio in liver DNA showed a profound reduction of mtDNA copy number (residual mtDNA levels were 5% of normal control levels). Analysis of the *POLG* gene sequences in blood DNA detected the p.R309H (c.926G>A) mutation in compound heterozygosity with the p.A889T (c.2665G>A) change (Figure 30).

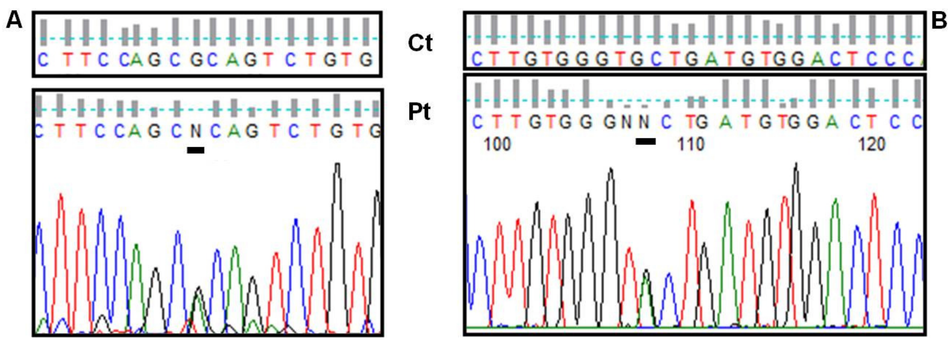


Figure 30 - Electropherograms of the *POLG* gene flanking the heterozygous mutations: A) p.R309H (c.926G>A) and **B)** p.A889T (c.2665G>A). The mutated bases identified in the patient (Pt) are underlined. Wild-type sequence in a control (Ct) is also shown.

P18, a 13-year-old boy, presented with myoclonic epilepsy, headache and bilateral high signal lesions of the occipital horns of the ventricles. Liver failure after treatment with valproate was documented, and he underwent liver transplantation. Multi-organ involvement and progressive course of the disease later manifested. The patient harbored a described homozygous p.A467T (c.1399G>A) mutation in *POLG* (Van Goethem *et al.*, 2001) (Figure 31). The mutation found in this patient represents the most common in the *POLG* gene, usually found in AHS disease with valproate induced hepatic failure. No mtDNA depletion or multiple deletions was found in muscle and liver in this patient. The parents of the index patient are both carriers of the same mutation confirming the Mendelian inheritance.

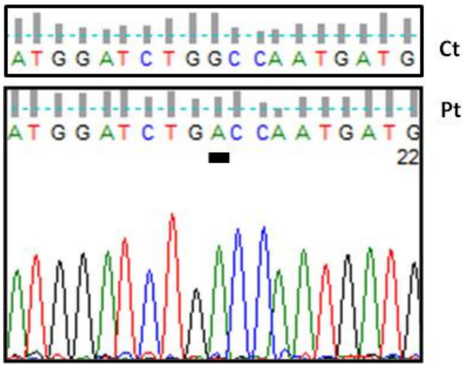


Figure 31 - Electropherogram of the *POLG* gene flanking the homozygous mutation p.A467T (c.1399G>A). The mutated base identified in the patient (Pt) is underlined. Wild-type sequence in a control (Ct) is also shown.

P35, a 4-year-old daughter of non consanguineous parents, presenting feeding difficulties, ventricular hypertrophy, retine alterations, low weight increasing and loss of consciousness. Low mtDNA levels were found in this patient (residual mtDNA levels were 15% of normal controls levels in muscle) and the molecular analysis of *POLG* gene revealed a reported mutation p.G268A (c.803G>C) in heterozigozity (Figure 32). No additional mutations, gross deletions or insertions were found by MLPA analysis, in the other allele of this patient. Thus other MDS genes are being investigated.

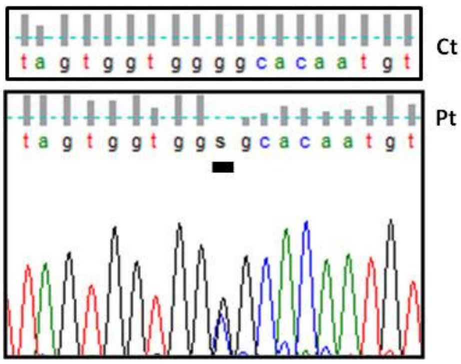


Figure 32 - Electropherogram of the *POLG* gene flanking the heterozygous mutation p.G268A (c.803G>C). The mutated base identified in the patient (Pt) is underlined. Wild-type sequence in a control (Ct) is also shown.

P42 is the youngest daughter born from healthy consanguineous parents (Figure 33A). Her older siblings (two sisters and one brother) are normal. The patient presented at age 4 months with psychomotor delay, hypotonia which evolved to spasticity, neurosensorial deafness and epilepsy. Urinary organic acids (GC/MS) showed the presence of 3-methylglutaric, 3-methylglutaconic, 3-hydroxysebacic, malic and fumaric acids and 3-hydroxyadipic acid lactone. There were increased levels of serum and CSF lactate and pyruvate whereas acylcarnitines were in the normal range. At age 5 months, spectrophotometric determination of MRC enzymes in a muscle biopsy showed a reduced activity of complex I (37% reduction as compared to the average value of age-matched controls). Histological studies showed predominance of type I fibers. The child died at age 10 months of cardiorespiratory arrest. Low mtDNA levels were found in this patient (residual mtDNA levels were 25% of normal age matched controls in muscle) and two reported mutations in *POLG* gene: p.G848S (c.2542G>A) in compound heterozygosity with the p.Q1236H (c.3708G>T), were found in the patient (Figure 33B and 33C), but not in her relatives P42a and P42b.

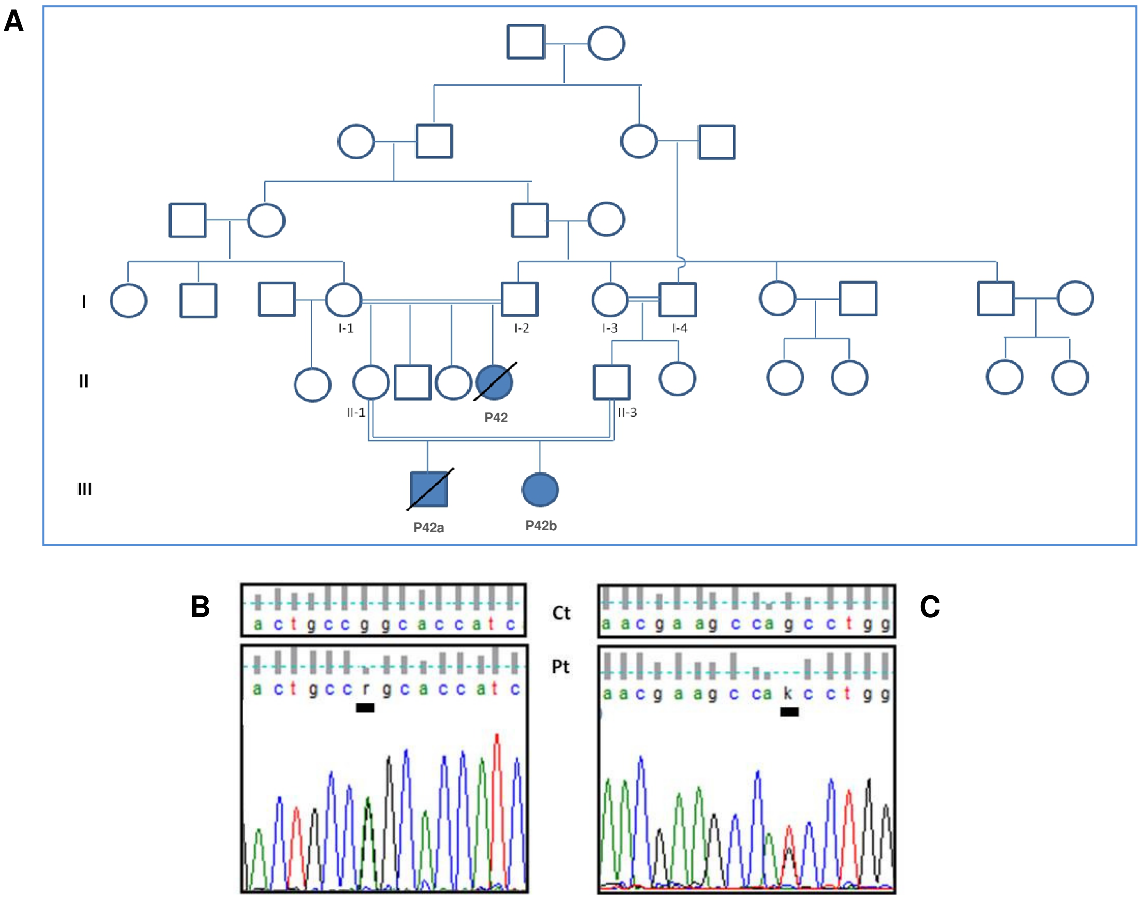


Figure 33 – A) Pedigree of P42 family; Electropherograms of the *POLG* gene flanking the heterozygous mutations: A) p.G848S (c.2542G>A) and **B)** p.Q1236H (c.3708G>T). The mutated bases identified in the patient (Pt) are underlined. Wild-type sequence in a control (Ct) is also shown.

P42a is the first son born to the eldest sister of case P42. The baby boy born from a consanguineous marriage and presented since the neonatal period with hypotonia, nystagmus and feeding difficulties. Urine organic acids showed the presence of 3-methylglutaconic acid. There was increased lactate in blood and in the CSF. At the age of 2 months, the patient presented with drug resistant seizures and died shortly after because of a cardiac failure, and therefore a muscle biopsy was not performed. Consequently mtDNA copy number was not performed, however the molecular study of *POLG* gene was performed in the DNA extracted from blood, but none of the mutations identified in his aunt were present. His younger sister, P42b, niece of P42, with 5-month-old, had since age 1 month a similar clinical picture, as well as a similar pattern of urinary 3-methylglutaconic acid production. OXPHOS studies in a muscle biopsy showed a reduced activity of complex I (38% reduction as compared to the average value of age-matched controls) and a severe mtDNA depletion was also found in muscle (residual mtDNA levels were 18% of normal age matched controls), although no mutations in *POLG* gene were identified.

TAZ

BTHS is an X-linked infantile-onset cardioskeletal disease caused by mutations in the *TAZ* gene. The majority of reported mutations are small insertions or deletions as well as missense mutations, although large deletions and truncating mutations have also been reported.

In this study we identified a patient (P38) who presented some of the reported clinical features associated with BS, such as: cardiomyopathy, skeletal myopathy, growth delay, neutropenia, with no urinary excretion of 3-methylglutaconic acid (3-MGCA), which must not be considered mandatory for a definitive diagnosis of BS. The patient also presented lactic acidosis since birth. MtDNA content was severely reduced in muscle tissue (residual mtDNA levels were 30% of normal age matched controls). Molecular analysis of the *TAZ* gene confirmed the clinical diagnosis by revealing a new missense mutation in hemizygosity: p.H176R (c.527A>G). This aminoacid substitution had not been found in 200 healthy control alleles. ClustalW analysis revealed a high degree of evolutionary conservation of histidine at residue 176 (His176) in the tafazzin protein (Figure 34). PolyPhen predicted that the mutation is probably damaging. This prediction was confirmed by ClustalW and SIFT analyses.

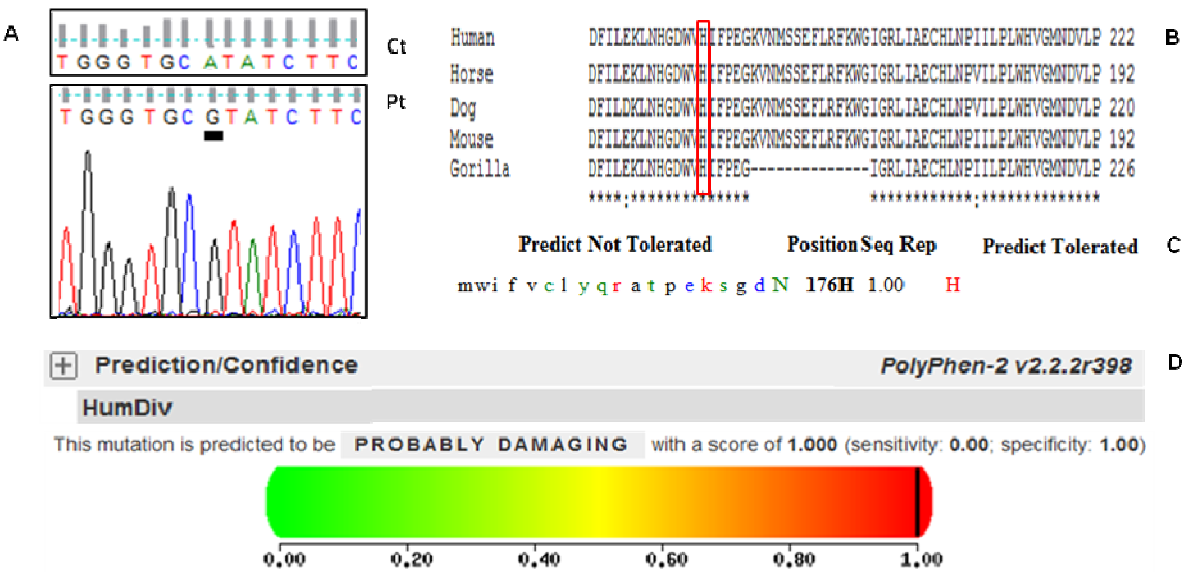


Figure 34 - Molecular analyses of a patient harboring a novel mutation in *TAZ*. A) Electropherograms of the *TAZ* gene flanking the heterozygous p.H176R (c.527A>G). The mutated base identified in the patient (Pt) is underlined. The wild-type sequence in a control (Ct) is also shown. **B)** ClustalW aligments; **C)** Sift predictions; **D)** PolyPhen-2 prediction of functional effects of human variations.

4.2. “Secondary” mtDNA depletion

We detected a mild-to-moderate mtDNA depletion in 11 individuals with neurometabolic diseases that secondarily impact on OXPHOS production or mtDNA levels. Few of those are worth reporting for their clinical importance, including four individuals in a family where we identified a clinical phenotype consistent with severe olivo-ponto-cerebellar atrophy, late-onset ataxia and psychosis together with a low complex III activity in muscle and a novel mutation in *TTC19*, and one patient with a deficiency in the mitochondrial arginyl-tRNA synthetase, caused by a mutation in *RARS2*.

TTC19

Autosomal recessive cerebellar ataxias are inherited disorders typically manifesting within the second decade of life, although metabolic forms of the disease might present at younger age. Atrophy of the cerebellum and brainstem are often prominent features, but other structures can be affected as well, leading to a broad range of clinical phenotypes.
CIII catalyzes the transfer of electrons from reduced coenzyme Q to cytochrome c. One of the 11 subunits, cytochrome b, is mtDNA-encoded. Mutations in the *MT-CYB* and *BCS1L* genes account for the vast majority of patients with CIII deficiency detected in skeletal muscle. It has recently been suggested that mutations in the human tetratricopeptide 19 gene (*TTC19*) may also be involved in the etiology of CIII deficiency, probably through impaired assembly of the holocomplex.
Four siblings in a consanguineous Portuguese family with CIII deficiency were investigated. Sequence analysis of genomic DNA was carried out to identify disease-causing mutations. Studies in muscle homogenate and skin fibroblasts from patients were also performed to investigate the functional consequences of the mutation on oxidative metabolism.
The propositus, P33 (II-01), (Figure 35A), was a 49-year-old man who had no clinically relevant complaints until the age of 27 years, when he manifested progressive back pain, clumsiness, gait difficulties, and a generalized anxiety disorder. Over the following years, additional psychiatric manifestations appeared, including major depression and visual hallucinations which did not respond to antipsychotic drugs. Neurological examination at age 37 revealed difficulties in tandem gait, loss of balance while climbing stairs, segmental ataxia, observable as dysmetria in the nose-finger and heel-shin tests, and interruptions in fast alternating hand movements. Dysarthric speech, dysphagia, and slow saccades were also detected. Subsequently, the patient displayed moderate spastic paraparesis with pyramidal tract signs affecting both the upper and the lower limbs. By the

age of 42, he presented severe muscle atrophy and, unable to walk or stand, was bedridden. His psychosis had worsened as a result of the onset of visual and acoustic hallucinations. He died of respiratory insufficiency aged 49 years.

Three additional sibs P33a (II-04), P33b (II-05), and P33c (II-06) showed an ataxic syndrome and psychiatric manifestations. The parents and two additional siblings (in their 50s), who were all asymptomatic, had no major neuropsychiatric complaints and a normal examination.

Brain imaging in all the patients at different ages showed a pattern of olivo-ponto-cerebellar atrophy (Figure 35B) and abnormal hypersignal in the caudate, putamen, medullary olives, cerebellar dentate nucleus and medial midbrain on T2-weighted MRI scans. Cortical brain atrophy (especially in the frontal lobe) appeared at a later age in patients P33 and P33a. No white matter changes or structural brain lesions were observed. Routine histochemical stains for oxidative metabolism in muscle biopsies were unremarkable in the four patients, whereas measurement of RC enzymes revealed a marked reduction of CIII activity in all of them (on average, residual activity corresponded to 33% of that recorded in age-matched normal controls).

A novel c.963_966delTGGC mutation in *TTC19* was identified (Figure 35C) in the propositus. This mutation predicts a frameshift and the synthesis of a truncated protein (p.A321Afs*8), lacking about 35% of its carboxy terminus. The new mutation was found to be homozygous in the four patients, heterozygous in their parents and in unaffected sibs, and absent in 300 ethnically-matched controls. The c.963_966delTGGC was expressed in tissues from patients P33 and P33c and the amount of mRNA/TTC19 transcript appeared to be semi-quantitatively reduced in muscle from case P33c (data not shown). Using BNGE followed by 2D-SDS and anti-Core2 antibody, there were nearly absent spots for CIII and CIII2+IV super-complexes in muscle from patient P33c compared to control muscle (Figure 35D), suggesting early impairment of assembly of CIII, as already outlined elsewhere (DiMauro *et al.*, 2005). By Western blotting, we found no protein using a specific anti-TTC19 antibody in muscle and fibroblasts from patients P33 and P33c (Figure 35E). We also observed a statistically significant increase ($p<0.03$) of ROS production in basal condition and after short-term H_2O_2 treatment (Figure 35F). Neither cellular ATP level nor ROS production were influenced by treatment with AICAR or bezafibrate, drugs known to enhance cellular oxidative metabolism.

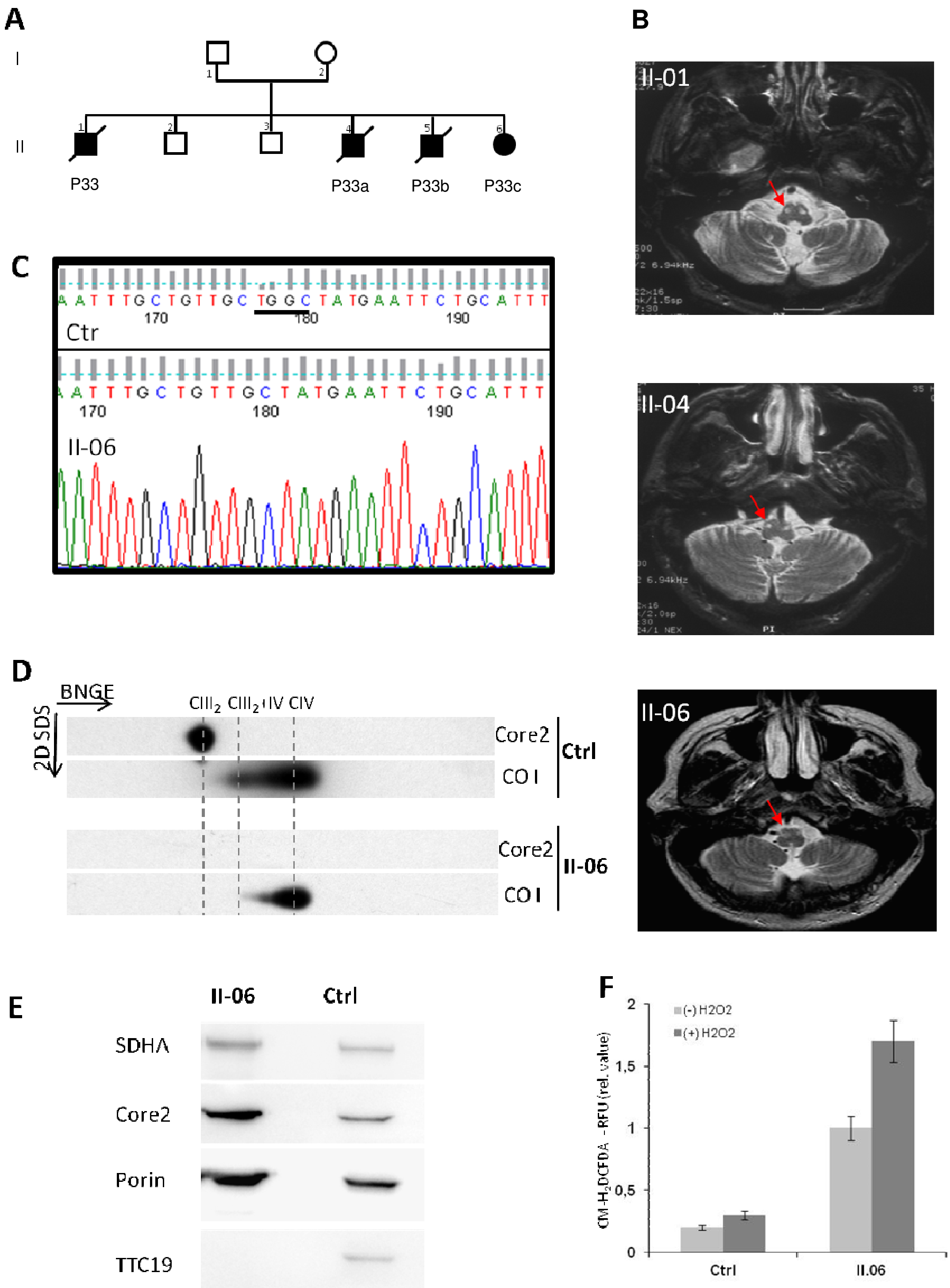


Figure 35 - Family pedigree, brain MRI and molecular studies in the new Portuguese kindred harboring a mutation in *TTC19*. A) Family tree. Square, men; circle, woman; filled symbols are affected individuals. Slash indicates deceased subjects. **B)** Brain MRI in three different affected patients (II-04, II-05, II-06) in this family. Arrows indicate pontine atrophy. **C)** Electropherograms of the *TTC19* gene flanking the homozygous c.962_967delTGGC (p.A321Afs*8) variant identified in patient II-06. Wild-type sequence in a control (Ct) is also shown. The deleted bases are underlined. **D)** BNGE followed by 2D-SDS-PAGE in muscle homogenates from patient II-06 and a control (Ctrl) using antibodies against the Core2 subunit to detect complex III and subunit I of cytochrome *c* oxidase (CO I) for complex IV. **E)** A representative immunoblot analysis of muscle homogenates from control (Ctrl) and patient II-06 using specific antibodies against subunits of complex II (SDHA), complex III (Core2), and TTC19. VDAC/porin was used to control for equal loading. Similar results were seen in cultured skin fibroblasts. **F)** Reactive oxygen species (ROS) production measured by CM-H_2DCFDA and expressed as relative fluorescent units (RFU) in skin fibroblasts from control (Ctrl) and patient II-06 incubated with or without with 1mM H_2O_2 for 30 min. Data are mean ± SD of three different determinations. Significance was set at $p<0.05$.

Interestingly, we also observed a moderate reduction of mtDNA copy number in muscle from P33c (residual mtDNA levels were, on average, 45% those of controls) (Figure 36A). The immunodetection pattern of the RC complexes was within normal values in skin fibroblasts (Figure 36B). A less profound defect for CIII and CIII2+IV super-complexes was seen in skin fibroblasts, by BNGE followed by 2D-SDS and anti-Core2 antibody (Figure 36C). ATP production was not reduced in either glucose or galactose medium in the patient cells (Figure 36D).

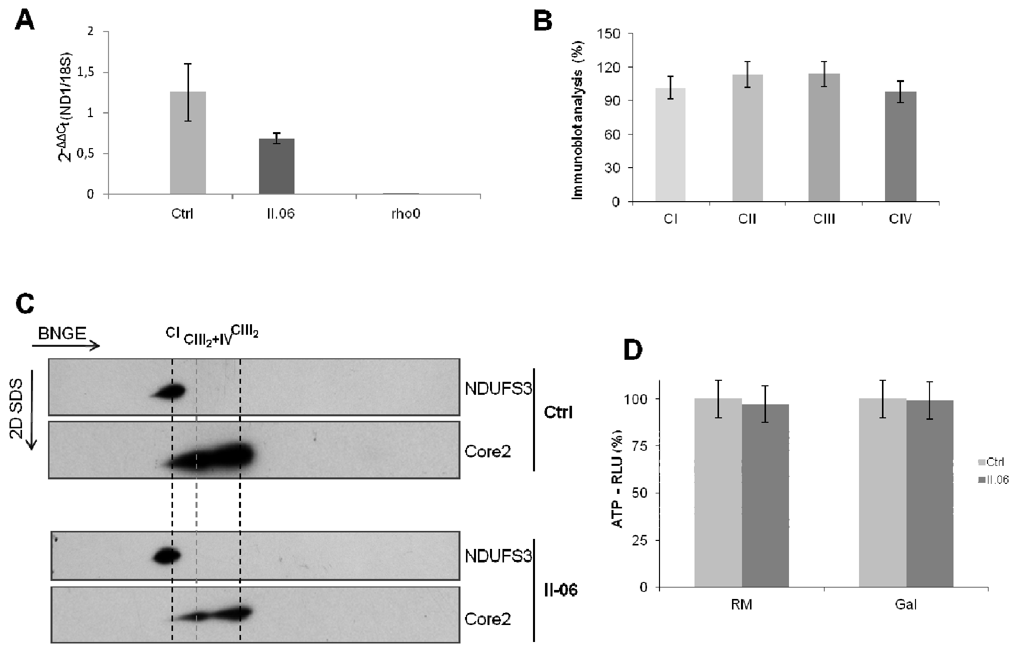


Figure 36 - Cellular and molecular analyses in a patient harboring a novel mutation in *TTC19*. A) Bar chart showing the relative quantification of mtDNA content by qPCR using the $2^{-\Delta\Delta Ct}$ method. The error bar in controls (Ctrl) indicates three times the standard deviation. Data represents the mean ± SD of three different determinations; rho0 are cells experimentally deprived of their own mtDNA. **B)** Bar chart showing the relative quantification of immunoblot analysis of skin fibroblasts from patient II-06 using specific antibodies against subunits of complex I (NDUFA9), complex II (α-SDHA), complex III (Core2), complex IV (COX II). Values are reported as the ratio to the internal control for loading (porin), and are expressed in reference to the average control value whose expression was arbitrarily attributed the value 1.0. Data are mean ± SD of three different determinations. **C)** BNGE followed by 2D-SDS-PAGE in patient II-06 and control (Ctrl) fibroblasts using antibody against the Core2 subunit to detect complex III (and supercomplexes III_2+IV), and an antibody against the subunit NDUFS3 of complex I. **D)** Luminometric measurement of ATP expressed as relative luminescence units (RLU), in skin fibroblasts from control (Ctrl) and patient II-06 cultured in either regular medium (RM) or medium supplemented for 72 h with galactose (GAL). Data represents the mean ± SD of three different determinations.

RARS

To guarantee fidelity in translation, it is important to attach the right amino acid to the tRNA, and to ensure that the tRNA recognizes, through its anticodon, the correct codon in the ribosomal A-site. Incorporation of an incorrect amino acid into the nascent polypeptide could cause misfolding and production of defective or dominant interfering proteins. Amino acids are attached to tRNAs by amino-acyl-tRNA synthetases, each of which is specific for a single amino acid. However, as there can be several codons and several different tRNAs for a single amino acid, an amino-acyl-tRNA synthetase can “charge” several different tRNAs. If this function were defective, certain codons would become ambiguous, resulting in the synthesis of misfolded proteins, which could aggregate to form inclusions and induce further protein misfolding. Misfolding and protein aggregation are thought to underlie several neurodegenerative disorders. This holds true for both cytoplasmic and mitochondrial protein synsthesis. In particular, mutations in *RARS2* are associated with severe encephalopathy with pontocerebellar hypoplasia.

In this study we identified a 4-year-old boy (P20) who presented early-onset, rapidly progressive cerebral and ponto-cerebellar atrophy with intractable epilepsy. Lactate was markedly elevated in blood and urine (three-fold normal values) but not in the CSF. At age 4 months, brain MRI showed marked cerebellar hypoplasia and atrophy and MR-S revealed an elevated lactate peak. At 1 year, head circumference dropped below the 3rd percentile. Lactic acidosis was no longer consistently detected in blood and urine. The patient continues to suffer from intractable multifocal seizures without improvement. Serial MRI scans performed at the ages of 12 months, and 2 years confirmed the progression to severe cortical and ponto-cerebellar atrophy (Figure 37).

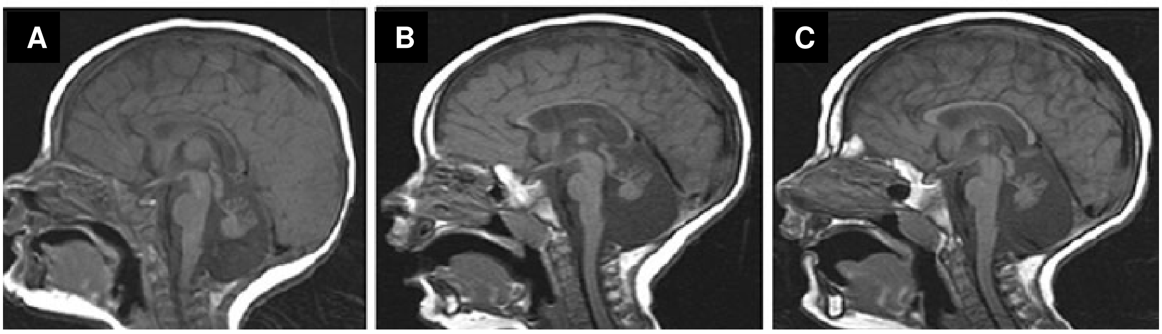


Figure 37 - Brain MRI (Sagittal T1 weighted serial images). These images were performed in P20 at age 4 months (A), 1-year (B) and 2-years (C) showing atrophy of the cerebrum, cerebellum and brainstem.

From these findings the disease appears to affect selectively the brain with first symptoms appearing in the neonatal period and manifesting with severe hypotonia, intractable seizures, and lactic acidosis. The EEG pattern consisted of diffuse slowing and bilateral, multifocal epileptiform abnormalities at onset with progressive deterioration. After the first year of life, the child showed very frequent myoclonic jerks in upper limbs.

Molecular investigations of *RARS2* disclosed in compound heterozygousity the p.Q12R/ p.W241R (c.35A>G/ c.721T>A) mutations (Rankin *et al.*, 2010; Namavar *et al.*, 2011). It has already been demonstrated that p.Q12R interferes with a splicing-enhancer element, producing abnormal splicing with retention of 221-bp of intron 1, resulting in frameshift with an expected protein truncation at residue 25 (p.Q12fsX25). The p.W241R mutation is predicted to be probably damaging upon SIFT, Polyphen2, and PMut (http://mmb.pcb.ub.es/PMut/PMut.jsp) analyses. None of the mutations identified in this study were detected in 200 healthy, ethnically-matched control chromosomes by sequencing (Cassandrini *et al.*, 2013).

Western blotting in skin fibroblasts showed that the level of the protein was reduced to 28 %, of the control mean value after normalization to the immunodetection of VDAC/porin (Figure 38A). A moderate reduction of mtDNA copy number was also observed in cultured skin fibroblasts from the patient (residual mtDNA levels were, on average, 43% those of controls) (Figure 38B) .

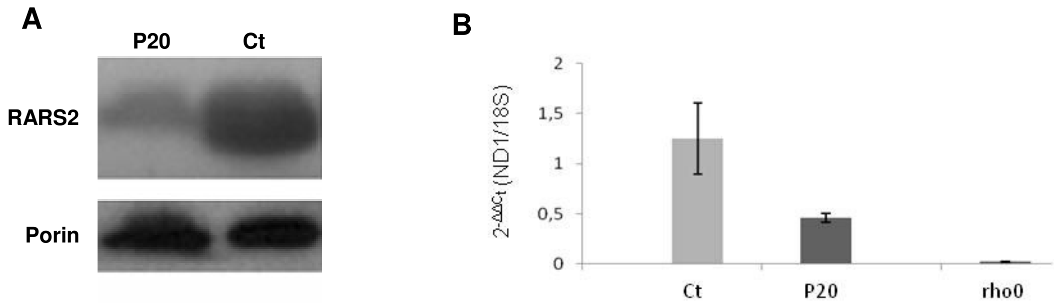


Figure 38 - Cellular and molecular analyses in P20, harboring a novel mutation in *RARS2*. A) A representative immunoblot analysis of fibroblasts homogenates from control (Ct) and P20 using specific antibodies against RARS2. VDAC/porin was used to control for equal loading. **B)** Bar chart showing the relative quantification of mtDNA content by qPCR using the $2^{-\Delta\Delta Ct}$ method. The error bar in controls (Ct) indicates three times the standard deviation. Data represents the mean ± SD of three different determinations; rho0 are cells experimentally deprived of their own mtDNA.

4.3. Multiple deletions

One of the patients analyzed in this study, whose clinical syndrome satisfied the criteria for MNGIE in association with weight loss and progressive gastrointestinal dysmotility, presented multiple mtDNA deletions together with a mutation in *TYMP*.

TYMP

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is a rare, autosomal recessive, multisystem and devastating disease that usually manifests in childhood and leads to death around age 35 years. MNGIE is caused by mutations in the *TYMP* gene encoding thymidine phosphorylase (TP). Loss of TP activity determines marked elevation in thymidine and deoxyuridine levels in body fluids, nucleotide pool imbalance, subsequent instability of mtDNA and impairment of the mitochondrial MRC. Among the important consequences of MNGIE syndrome, multiple deletions and/or depletion of mtDNA have been observed.

We studied a 50-year-old patient (P36) who had complained of intermittent postprandial vomiting and gastrointestinal dysmotility with episodes of diarrhea and constipation leading to progressive weight loss over few years. Neurological examination showed severe cachexia and numbness distally in the four limbs, mild generalized weakness and diffuse muscle atrophy. Multiple mtDNA deletions were also detected by qPCR.

Molecular analysis of the *TYMP* gene in muscle DNA from P36 revealed the p.S160P (c.478T>C) mutation in compound heterozygosity with the p.A465T (c.1393G>A) mutation, both already described in the literature (Nishino *et al.*, 2000; Kocaefe *et al.*, 2003) (Figure 39).

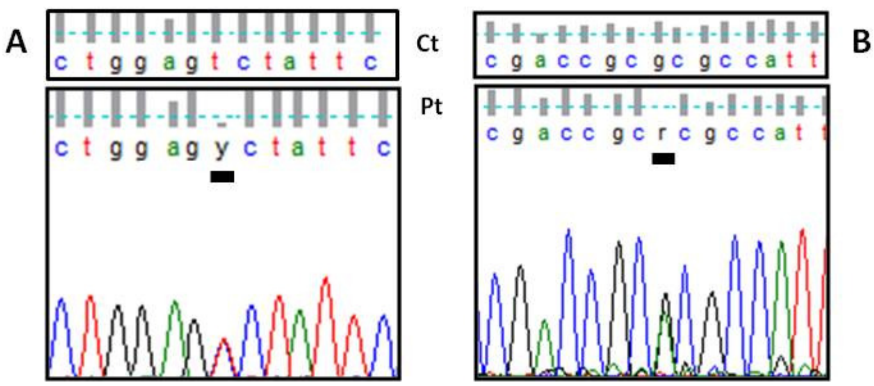


Figure 39 - Electropherograms of the *TYMP* gene flanking the heterozygous mutations: A) p.S160P (c.478T>C) and **B)** p.A465T (c.1393G>A). The mutated bases identified in the patient (Pt) are underlined. Wild-type sequence in a control (Ct) is also shown.

Additionally a novel variant p.M74K (c.221T>A), presented in heterozygosity, was also identified in this patient (Figure 40A). This variant has not been found in 200 healthy control alleles. ClustalW analysis revealed a high degree of evolutionary conservation of metionine at residue 74 in the TP protein (Figure 40B). Bioinformatic analysis using Sift and Polyphen2 showed that the mutated aminoacid is not tolerated and that the mutation is probably damaging. (Figure 40C, 40D).

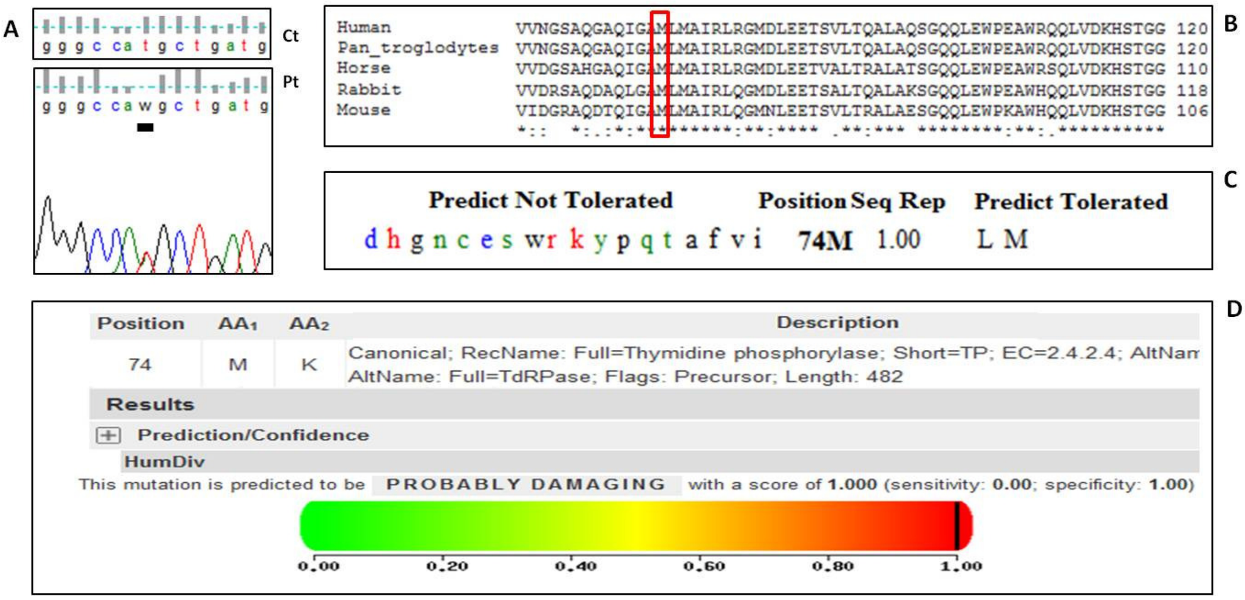


Figure 40 - Molecular analyses of a patient harboring a novel mutation in *TYMP*. A) Electropherograms of the *TYMP* gene flanking the heterozygous p.M74K (c.221T>A). The mutated base identified in the patient (Pt) is underlined. The wild-type sequence in a control (Ct) is also shown. **B)** ClustalW aligments; **C)**Sift predictions; D**)** PolyPhen-2 prediction of functional effects of human variations.

CHAPTER V

DISCUSSION

DISCUSSION

A mitochondrial disease manifesting at birth or soon after is more likely to be associated with nDNA than with mtDNA mutations, but until very recently, our lack of knowledge regarding the mechanisms underlying mitochondrial gene transcription and translation and the complex interaction between the "two genomes" has limited the diagnostic power. mtDNA deletion and depletion syndromes, and disturbances in the mitochondrial translation machinery have increasingly emerged as a major cause of a wide spectrum of infantile and childhood-onset multisystem disorders. Depletion syndromes could result from any imbalance of the mitochondrial dNTP pools available for mtDNA replication, as well as abnormalities in either the mitochondrial helicase or DNA polymerase. Consistent with the different phenotypes, mtDNA depletion may affect specific tissue (most commonly, the brain and muscle or liver) or multiple organs, including heart and kidney. More than 75% of these patients had onset during the first year of life, and the disease was rapidly fatal in most cases (Hirano *et al.*, 2001, Sarzi *et al.*, 2007). Moreover, though the components of the complicated mitochondrial protein-synthesis machinery are exclusively nuclear encoded, the majority of mutation affects correct translation of mtDNA encoded subunits of the OXPHOS and arises from a still undetermined number of genetic defects. Indeed, there is still limited information on the many mitoribosomal proteins; the several tRNA maturation enzymes; the aminoacyl-tRNA synthetases; the translation initiation, elongation, and termination factors; and the predictably larger number of unidentified factors needed for ribosome assembly (Jacobs *et al.*, 2005, Smits *et al.*, 2010).

Expansion in the number of proteins regulating mitochondrial diseases and associated genes are going hand in hand with an increasing genetic and clinical phenotypic heterogeneity of this group of disorders. Identifying the causative genes is important not only to allow adequate antenatal options, family planning and prenatal diagnosis, but also to improve understanding of the disease pathophysiology and, therefore to envisage the therapeutic options. The elevated number of genes found to be involved is a driving force for the development of high throughput strategies. The recent advances in sequencing technology will facilitate the molecular investigations of genes associated with mtDNA disorders in general. Reports concerning the use of next generation sequencing (NGS) for the diagnosis of mitochondrial disorders are already emerging (Vasta *et al.*, 2009; Calvo *et al.*, 2012; Haack *et al.*, 2012). A recent one proved the efficacy, in clinical diagnosis, of the use of target NGS for mitochondrial disorders; indeed, a clear molecular etiology was found in 55% of the patients studied by some groups (Calvo *et al.*, 2012). Since NGS is becoming a common option in neurogenetic disorders (Shendure *et al.*, 2012), it holds the promise to identify a greater number of patients with mitochondrial disorders as well (Dündar *et al.*, 2012). This would likely resolve some of the open issues emerging from the clinic, including difficult diagnosis, uncertain or unavailable genetic counseling and prenatal diagnoses, and unpredictable prognoses.

In this work, 47 of 90 cases, suspicious of disorders associated with mtDNA depletion or multiple deletions, as well as with biochemical and suggestive clinical features, were eligible for this study. In 55% of patients (26/47) we identified mtDNA depletion or multiple deletions; 65% of these patients presented "primary" mtDNA depletion, 31% "secondary" depletion and 4% mtDNA multiple deletions. The remaining 45% of the patients (21/47) were considered eligible for this study based on their suggestive clinical features (Figure 41).

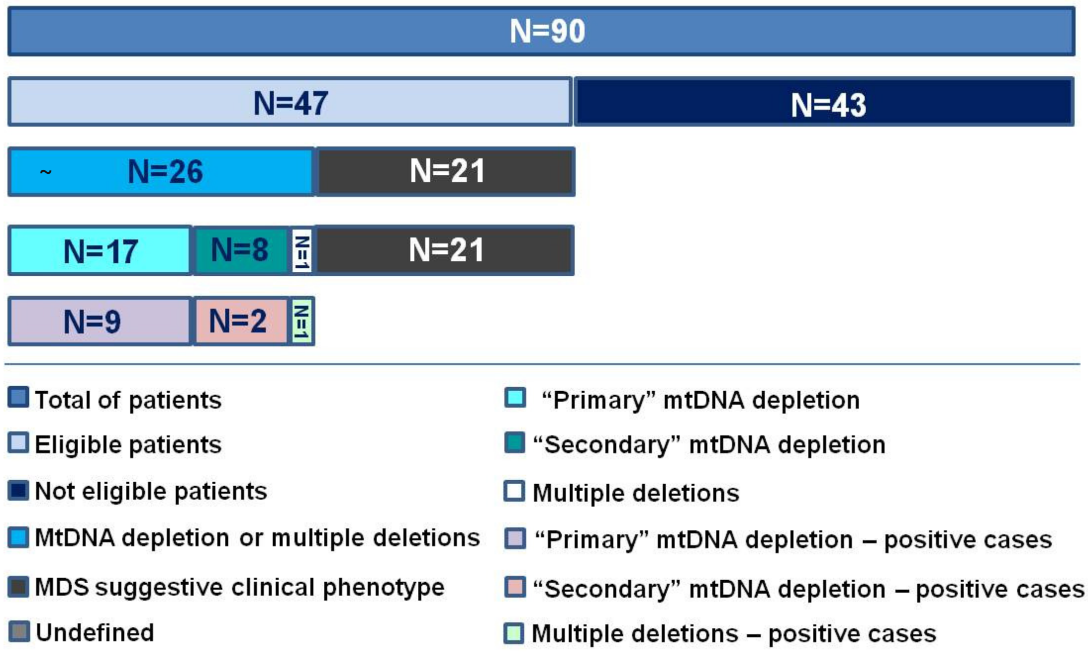


Figure 41 - Schematic representation of the group of patients investigated in this study.

A positive molecular result was obtained in 25% of total eligible patients (12/47): 10 were part of the group with mtDNA depletion or multiple deletions (N=26), and only two were from the other group of patients (N=21). Most of the patients in this study (35/47) remained with an undetermined molecular diagnosis.

Identification of a positive molecular etiology varied between groups with mutations found in “primary” mtDNA depletion (75%), “secondary” mtDNA depletion (17%) and mtDNA multiple deletions (8%) (Figure 42). We also considered two patients from the group of MDS suggestive clinical phenotype (N=21) as presenting “primary” mtDNA depletion, due to their typical clinical phenotype associated with mutations in *DGUOK* and *POLG* genes (Villarroya *et al.*, 2009; Van Goethem *et al.*, 2001). One of these patients, P18, was tested for mtDNA depletion and multiple deletions, however did not present any of these alterations, in the studied tissues.

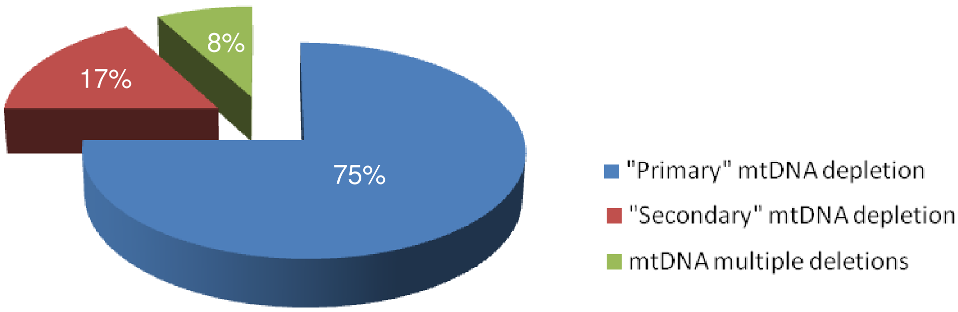


Figure 42 - Schematic representation of the positive molecular patients.

The spectrum of identified mutations found in patients with “primary” mtDNA depletion encompasses the following genes: *MPV17* (P15), *DGUOK* (P11), *SUCLA2* (P27), *C10orf2* (P32), *POLG* (P42, P9, P35, P18), and *TAZ* (P38), while those with “secondary” mtDNA depletion include: *TTC19* (P33) and *RARS2* (P20). Finally, we identified a mutation in *TYMP* in a patient with mtDNA multiple deletions (P35).

The group of “primary” mtDNA depletion showed a high positive molecular rate (75%) in the most common genes associated with MDS, with the exception of P38 harboring a mutation in *TAZ* gene. The positive cases will be further discussed below.

MPV17

To date a total of 31 patients have been reported with *MPV17* mutations, and here we describe a new Brazilian patient with fatal hepatocerebral presentation of MDS. MtDNA depletion was confirmed by comparing patient’s mtDNA copy number with tissue and age matched controls, being 98% and 77% in patient’s liver and muscle, respectively.

Molecular studies identified a homozygous c.186+2T>C mutation in *MPV17* gene, only been described in one previous case in the heterozygous state with a “little” better prognosis (El-Hattab *et al.*, 2010). This mutation is located at the invariant splice donor site and is predicted to abolish the splicing donor site of exon 3 (http://www.cbs.dtu.dk/services/NetGene2/ and http://www.fruitfly.org/seq_tools/other.html). Although the parents were non-consanguineous, they were from the same Brazilian region and the presence of a homozygous mutation suggests that the parents shared a common ancestor or that this recessive mutation has high population prevalence in the Brazilian population.

In this study, we identified for the first time a Brazilian (P15) with a *MPV17* splicing mutation in a homozygous state. Our study contributed to expand the spectrum of patients with *MPV17* mutations, and will be important for an accurate genetic counseling and a prenatal diagnosis to the affected family.

DGUOK

In one case (P11) with suspected hemochromatosis we found a homozygous mutation in *DGUOK*. In principle, an excess of free iron ions may have deleterious toxic influence on the liver and subsequent organ damage (Visapää *et al*. 2002; Ramm and Ruddell 2005).

An impaired function of the mitochondria associated with dGK deficiency may still aggravate the process. Decreased hepatic ATP content and reduced activity of the cytochrome *c* oxidase was observed in an experimental model of iron overload (Bacon *et al.*, 1993). In one report describing a child with iron storage disease, a severe depletion of mtDNA (9% of the normal value) was identified (Vu *et al.*, 2001).

A better understanding of the primary or secondary factors influencing liver damage in MDS (including hypoglycemia and iron overload) may contribute to the improvement of the short- and long-term prognosis in children with the hepatic form of this mitochondrial disorder.

SUCLA2

We reported a new case (P27) of “mild” MMA with mutations in *SUCLA2*. The clinical and biochemical phenotype of our patient is strikingly similar to patients harboring mutations in *SUCLA2* previously reported in larger series (Carrozzo *et al.*, 2007). The mild elevation of the urinary levels of methylmalonate and succinylcarnitine raised the suspicion of a succinyl-CoA synthetase disease, which was confirmed by the identification of a novel mutation and the presence of mtDNA depletion, although not in high percentage in the fibroblasts, as expected in the muscle tissue.

Thus, our study shows the first SCS deficiency in the Portuguese population. The novel mutation meets several canonical criteria for pathogenicity. An additional interesting observation arising from our study relies on the reduction on ROS production upon AICAR treatment in cells from a *SUCLA2* patient. AICAR has already been found to improve growth and ATP content while decreasing ROS production in patients presenting mutations in subunits of the respiratory complexes I and IV (Golubitzky *et al.*, 2011) and is reported to fight the pathological mechanisms in a mouse model of mitochondrial encephalomyopathy (Viscomi *et al.*, 2011) AICAR as a pharmacological activator of AMP activated protein kinase (AMPK) might play a key role in the regulation of energy homeostasis, increasing mitochondrial biogenesis without altering mitochondrial membrane potential (Golubitzky *et al.*, 2011). If induction of mitochondrial biogenesis has a "therapeutic" role or influences mechanisms fighting apoptotic cell death (Dam *et al.*, 2013) will require further investigation.

C10orf2

We also identified a new case (P32) harboring mutations in *C10orf2*. It is unclear why some mutations in the *TWINKLE* gene cause autosomal recessive diseases (in the form of IOSCA syndrome or as a hepatocerebral/hepatocerebrorenal syndrome), whereas others result in autosomal dominant transmission of a disorder confined to extraocular muscles. However, a similar condition occurs in the syndromes associated with mutations in *POLG*, the DNA polymerase that is closely associated with the Twinkle helicase to control mtDNA replication. As similar reports (Dundar *et al.*, 2012), it seems that our study suggests to consider IOSCA in the differential diagnosis of neurometabolic disorders even outside Finland.

Our study also expands the background of MDS patients with uniparental dissomy (UPD) (Douglas *et al.*, 2011; Haudry *et al.*, 2012) and illustrates the need to consider UPD in cases of homozygosity of a rare mutation being responsible for recessive disease in non-consanguineous families. We report the first UDP of chromosome 10 in a patient presenting mutations in *C10orf2*.

Because the recurrence risk for UPD is very low, the risk for disease in further offspring for this couple is negligible (Haudry *et al.*, 2012).

POLG

We studied a patient (P9) with a severe clinical phenotype who died due to valproate induced fatal acute liver failure. Analysis of the mtDNA content revealed a severe depletion in liver (approx. 95%) and a heterozygous compound in the *POLG* was identified [p.R309H (c.926G>A); p.A889T (c.2665G>A)].

Several inborn errors of metabolism are known to represent a risk factor for severe idiosyncratic reactions to valproate, including liver toxicity. Many studies have focused on the interaction between valproate and mitochondrial function in general and mitochondrial disorders such as AHS in particular, as conditions predisposing to severe valproate toxicity. Recent studies gave evidence that *POLG* mutations can lead to a range of clinical phenotypes which predispose to the development of fatal liver failure after exposure to valproate. Nevertheless, a single case report suggests that there may be mutations in the *POLG* gene associated with reversible hepatotoxicity (Schaller *et al.*, 2011).

Another mutation commonly found in *POLG* and associated with valproate induced hepatic failure in AHS patients is the p.A467T (c.1399G>A). This condition was observed in P18. However, no mtDNA depletion or multiple deletions was found in muscle and liver from this patient. The cellular mtDNA content may be an indicator of the underlying molecular mechanism linking genotype to phenotype and explaining the patient's acute liver failure (Schaller *et al.*, 2011).

We also described in P35 in which a severe reduction od mtDNA copy number, but only one *POLG* mutation, p.G268A (c.803G>C), could be identified in heterozygousity. This mutation is located in the 3'->5' exonuclease region and is associated with the proofreading activity of POLG. It seems to act in a recessive way, unless a different genetic background may act as a modifier factor. In homozygosity usually causes the accumulation of mtDNA deletions (Graziewicz *et al.*, 2006). As no additional mutations, gross deletions or insertions were found in the other allele of this patient, other MDS genes, such as *TK2*, *RRM2B* and *C10orf2*, are being investigated.

We also investigated a family where the affected individuals have in common an abnormal pattern of urinary organic acids, with the presence of 3-methylglutaconic acid. At age of 4 months P42 presented psychomotor delay, hypotonia, neurosensorial deafness and epilepsy. The spectrophotometric determination of MRC enzymes in a muscle biopsy showed a reduced activity of complex I (37% reduction as compared to the average value of age-matched controls) and histological studies showed predominance of type I fibers. Patient 42a and 42b, her relatives, presented with a similar clinical phenotype, however the spectrophotometric determination of MRC enzymes was not performed in P42a because skeletal muscle biopsy was not available and P42b also showed a complex I deficiency. SDS-PAGE and BN-PAGE showed decreased levels of several subunits of complex I and decreased amount of fully assembled complex I in P42. As *POLG* is one of the essential factors required for mtDNA replication (Ropp and Copeland 1996), it is tempting to speculate that variations in this gene might impair mtDNA replication, affecting the synthesis of ND subunits. This ultimately could affect the ND subunit pools, that are believed to exist, and consequently affect complex I assembly due to its dependence on ND subunits to form the membrane arm (Ugalde *et al.*, 2004).

A significative reduction of mtDNA copy number was also detected in P42 and in P42b. In case 42 but not in 42a and 42b, we identified two mutations in *POLG*: p.G848S (c.2542G>A) and p.Q1236H (c.3708G>T). Mutations in this gene could affect the catalytic activity, the processivity of the enzyme or the the fidelity of mtDNA replication (Chan and Copeland 2009). However, due to the high consanguinity, this family could have two different disorders, what explains the fact that the molecular characterization of P42 relatives remain indeterminated. As these affected individuals have in common an abnormal excretion of 3-methylglutaconic acid, and there are different etiologies of 3-methylglutaconic aciduria syndromes, associated with OXPHOS dysfunction, this should be taken into account in further investigations.

TAZ

We identified in P38 a new hemizygous missense mutation, p.H176R (c.527A>G), associated with a significant reduction of mtDNA copy number in patient 38, who presented a clinical phenotype of BTHS.

Fewer than 200 living males are known worldwide, but evidence is accumulating that the disorder is substantially under-diagnosed (Clarke *et al.*, 2013). Allelic heterogeneity and the intrafamilial variability of expression are high. Thus, no genotype-phenotype correlation has been observed in any patients (Rigaud *et al.*, 2013).

The new mutation identified in this study contributed to expand the spectrum of patients with *TAZ* mutations, and will be important for an accurate genetic counseling and a prenatal diagnosis to the affected family.

Management of these patients includes medical therapy of cardiomyopathy, cardiac transplantation, antibiotic prophylaxis and granulocyte colony-stimulating factor therapy. Multidisciplinary teams/clinics are essential for minimising hospitalizations and allowing many more individuals with BTHS to live into adulthood (Clarke *et al.*, 2013).

In the group of “secondary” mtDNA depletion the positive rate was 17%, in genes that are associated with the assembly of the MRC complexes, in this case assembly of CIII, and mitochondrial translation. These cases will be discussed in the following parahraphs.

TTC19

In the course of our study we investigated a kindred with a “secondary” mtDNA depletion where four siblings, showed a novel mutation in *TTC19*. The Portuguese family we described is additional evidence that it is difficult to predict the onset (timing and symptoms) and progression of a neurodegenerative disease due to defective oxidative metabolism even when multiple cases have already occurred. In the family described herein, the age at onset and pattern of progression varied significantly, with P33a and P33b manifesting symptoms in their teens and showing a rapid downhill course, and P33 and P33c showing a later onset with a progressive disease course. In P33c, neuropsychiatric features appeared in spite of annual monitoring and attempts to treat the symptoms. Second, our data add to the relatively limited array of variants in genes related to CIII deficiency. Like the previously described *TTC19* mutation, the new c.963_966delTGGC seems to lead to a significant reduction of CIII holoenzyme, and impairment of early assembly steps, or overall stability, of the holoenzyme. However, the mechanism by which lack of TTC19 and the ensuing biochemical features affect cortical and deep brain neurons and lead to the clinical manifestations seen in our family remains at large unexplained.

It is tempting to hypothesize that the high levels of ROS production seen in cells from *TTC19* patient are even higher in selective brain structures probably because lack of functional components of CIII holoenzyme (for example, RISP) that are usually assembled in later stages. Interestingly, severe ROS damage, impaired motor coordination, and neurodegeneration in limbic system structures have been observed upon ablation of RISP in a brain-specific mouse model of CIII deficiency.

Summarizing, we described the first family outside Italy with a mutation in *TTC19*. The novel change manifested as a disorder characterized by degeneration of olivary-cerebellar neurons and a progressive psychiatric syndrome, findings that should alert clinicians when searching for additional cases.

RARS2

Infantile-onset diseases due to mutations in nuclear genes encoding proteins targeted to the mitochondria are more common than primary mutations in the mitochondrial genome itself and their number increases at a rapid pace (McFarland and Turnbull 2009; Chrzanowska-Lightowlers *et al.*, 2011). A mutation in any of these genes has the possibility to produce severe biochemical defects of OXPHOS or be harmful for mtDNA maintenance, or both. As a consequence, clinical presentations may vary but patients, especially infants and children, frequently suffer from severe neurological illness because the developing brain has the highest request in oxidative substrates for functioning.

A similar pattern of early-onset epileptic encephalopathy and progressive cerebello-cerebral encephalopathy are common to one of the studied patients (P20) and represent useful clues for screening *RARS2* particularly if associated with early lactic acidosis and lactate peak at MR-spectroscopy. Early onset seizures have also been observed in previously reported *RARS2* patients (Edvardson *et al.*, 2007; Rankin *et al.*, 2010; Namavar *et al.*, 2011; Glamuzina *et al.*, 2012) but lactic acidosis may be overlooked as disease progresses and its absence should not preclude *RARS2* testing.

At the long-term follow-up, this patient showed progressive microcephaly, spastic quadriplegia, and virtual absence of psychomotor development. Gastroesophageal reflux was also particularly severe in this case as disease progressed. Considering that the first signs of encephalopathy such as diffuse hypotonia and poor eye contact were already present in the neonatal period, as well as a relative microcephaly at birth, it seems obvious that the genetic condition has a prenatal onset in most patients and affected mostly the brain. Follow up MRI revealed ensuing cerebral cortical atrophy together with progressive pontocerebellar atrophy and confirmed described aspects of white matter "depletion" (Edvardson *et al.*, 2007).

Given the clinical and characteristic neuroimaging pattern related to mutations in *RARS2* the differential diagnosis with other neurodegenerative conditions starting in early infancy such as neuronal ceroid-lipofuscinoses and AHS is not complex.

Lending further evidence to the heterogeneity of clinical presentation of infants with mitochondrial encephalopathies, this work has expanded the array of disease-causing variants in *RARS2*, offering clues to recognize further similar patients.

The methodology of qPCR also enabled the detection of mtDNA multiple deletions, which was the case of P36, whre we found mutations in the *TYMP* gene.

TYMP

In this study we identified a MNGIE patient (P36) with mtDNA multiple deletions. Molecular analysis of the TYMP gene revealed the presence of three heterozygous mutations two of them already described in the literature [p.S160P (c.478T>C) and p.A465T (c.1393G>A)] and a novel variant [p.M74K (c.221T>A)], probably pathogenic. In this case a familial investigation should be performed to clarify the segregation of the alterations found in this patient.

The poor quality of life and early death in patients with MNGIE demand urgent treatments, but so far, therapy has been largely supportive, including total parenteral nutrition, pain relief, and treatment of infections. Abdominal pain is often treated with some success by celiac plexus neurolysis. Hemodialysis is another strategy but re-accumulation of the nucleosides is too rapid. Recently, allogenic stem cell transplantation and platelet infusion have been proposed and though showing promising results they require longer follow up.

DISCUSSION HIGHLIGHTS

The group of studied patients suspicious of cross-talk disorders was investigated at a molecular level based on their suggestive clinical features and/or the presence of mtDNA depletion or multiple deletions.

In this study, using a classical molecular approach (PCR followed by Sanger sequencing), we have successfully characterized 25% (12/47) of the studied patients, a number that is in accordance with recent reports using novel sequencing technologies (Calvo *et al.*, 2012; Lieber e*t al.*, 2013). The majority of mutations was found in genes directly involved in the mtDNA maintenance. Furthermore, identification of these novel patients has contributed to expand the molecular spectrum of these diseases, as six novel mutations were identified. These molecular results will also be important to offer an accurate genetic counseling and a prenatal diagnosis to the affected families.

However, a significant number of eligible patients (35/47) still remain without molecular characterization. The use of targeted NGS of mitochondrial proteins has shown the efficacy of the new methodology to amplify the rate of molecular diagnosis as well as to find new genes related to MDS (Lieber e*t al.*, 2013). Furthermore, several lessons can already be taken from the incorporation of NGS in the clinic, such as: i) targeted exome sequencing is a cost-effective and time-efficient alternative to traditional, sequential testing of the mtDNA and individual nuclear genes. For a subset of patients, a firm molecular diagnosis can be established quickly in a minimally invasive manner; ii) there is a great phenotypic overlap between mitochondrial disorders and other genetic syndromes limiting the rate of detection of confirmed mutations, and, as costs decrease, this argues for whole exome or whole genome sequencing, and iii) when possible, familial sequencing can be performed to facilitate phasing of haplotype, detection of *de novo* variants, and filtering of candidate variants. These features demonstrate that targeted exome sequencing could be an effective alternative to the sequential testing of mtDNA and individual nuclear genes as part of the clinical investigation of mitochondrial disease.

The final aim of this study was to address therapeutical options for mitochondrial disorders. We have selected some compounds from the literature, such as AICAR, bezafibrate and riboflavin, that were tested in other groups of mitochondrial diseases. Upon bezafibrate and riboflavin treatment of cells from *SUCLA2* and *TTC19* patients no significant changes were observed in ROS content between treated and untreated cells. However, bezafibrate was beneficial for increasing ATP content in both patient cell lines. For AICAR treatment, the results obtained showed a reduction in ROS production in the same cell lines. AICAR has already been shown to improve growth and ATP content in cells from patients harboring mutations in subunits of the MRC complexes I and IV and it is said to fight the pathological mechanisms in a mouse model of mitochondrial encephalomyopathy (Viscomi *et al.*, 2011). Our data indicate that AICAR has the possibility to raise ATP production and counteract ROS production even in cases with impaired mtDNA biogenesis. Whether stimulation of mitochondrial biogenesis has a “therapeutic” role or influences mechanisms fighting apoptotic cell death will require further investigation. Screening of potential therapeutic compounds for mitochondrial diseases is a challenge and a hope for the patients carrying these devastating diseases.

CHAPTER VI

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The investigation carried out in this work, using a combination of clinical, biochemical, neuroradiological, pathologic, and molecular information, has further reinforce the need for a multidisciplinary methodology to establish the basis of intergenomic communication defects.

From a general point of view, a good approach to study patients suspicious of intergenomic communication diseases could pass through: i) a better clinical selection of patients, which may range from well-defined syndromes to non-specific multisystem phenotypes, in which neurological involvement is usually present; ii) a detailed biochemical data, such as lactate, pyruvate, alanine and organic acid profiles, as well as neuroimaging and pathological findings, are also important clues for the diagnosis of these disorders; iii) determination of MRC complexes enzymatic activity, as mtDNA encodes subunits of CI, CIII, CIV and CV and a mtDNA depletion can be expected to cause a combined deficiency of all, but not complex II, as it is not mtDNA-encoded. However, this result can be normal, if skeletal muscle is not among the affected tissues, e.g., brain or liver; iv) determination by real-time qPCR of mtDNA depletion and multiple deletions; and v) identification of the causative genes. All of these features are represented in the diagnostic algorithm illustrated in Chapter III, based on our laboratorial experience and practice.

The molecular etiology of the disease in most patients presenting mtDNA copy number reduction remains unknown. This suggests that in perspective, a wider use of next generation high-throughput strategies, such as Targeted MitoExome Sequencing, Whole Exome Sequencing (WES) and/or Whole Genome Sequencing (WGS) are likely to provide advantages in terms of screening of individual genes (including the “tiny” mtDNA), definition of new etiologies, and benefit for a larger number of patients, as demonstrated by the analysis of a representative cohort of 291 unrelated infantile patients with “definite” OXPHOS disease from the Murdoch Childrens Research Institute. However, 124 cases had previous molecular diagnosis, which suggests that MitoExome sequencing could enable diagnosis in roughly 47% of all infantile patients and prioritize candidates in a further 20% of cases (Calvo *et al.*, 2012).

The identification of new disease-related genes open the possibility to study their functions and to understand their pathogenetic mechanisms, which will lead to fundamental advances in our understanding of mitochondrial biology and hopefully provide insights towards novel therapeutic strategies. On the other hand, a larger outlook of the MitOME (the set of proteins predicted to be target to mitochondria) expand the possibility of variants of unknown significance (VUS) and require more robust and relatively simpler functional methods for validation. WES or WGS might provide on the biology of communication between the two genomes and also on the possibility that also point mutations in mtDNA (and not only depletion or multiple deletions) might be effect of variants in nuclear genes.

The use of a model organism, such as zebra fish, will be also powerful to analyze disease significance and functional consequences of variations detected in genes involved in the intergenomic communication disorders. Promoting the use of this system in combination with more traditional *in vitro* experimental models will offer the opportunity not only to investigate genes harboring VUS (and attribute their pathogenicity) but also to improve our knowledge on the level of functional complementation between two apparently different cell systems.

Moreover we expect to develop and validate a cell-based, high-throughput screening assay to search for new therapeutically active molecules, assessing not only ATP production but also potential oxidative damage to the cell. The disturbance in the pro-oxidant-antioxidant balance in favor of the former is defined as oxidative stress, which is present in many inborn errors of metabolism, causing the accumulation of toxic metabolites or their precursors and leading to an increased free radical production (Wajner *et al.*, 2004). Cells can protect against oxidative stress through non-enzymatic and enzymatic antioxidants defenses. Thus, testing potencial therapeutical compounds in patient's cells could have an impact on patient’s health and advance new opportunities in personalized medical treatment of mitochondrial disorders.

CHAPTER VII

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REFERENCES

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- OMIM - http://www.ncbi.nlm.nih.gov/omim
- PMut - http://mmb.pcb.ub.es/PMut/PMut.jsp
- Polyphen2 - http://genetics.bwh.harvard.edu/pph2/
- Primer 3.0 - http://frodo.wi.mit.edu/primer3/
- SIFT - http://sift.jcvi.org/

CHAPTER VIII

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL

8.1 Real-time qPCR primers

Gene	Forward 5'-3'	Reverse 5'-3'
MT-ND1	CCCTAAAACCCGCCACATCT	GAGCGATGGTGAGAGCTAAGGT
MT-ND4	CCATTCTCCTCCTATCCCTCAAC	CACAATCTGATGTTTTGGTTAAACTATATTT
18S rRNA	GGCGTCCCCCAACTTCTTA	GGGCATCACAGACCTGTTATTG

8.2 PCR Reactions Mix

A. Reaction Mix for PCR

Reagents	Volume
ImmoMix Red®	6,25 µL
Primer Forward (10 pmol/µL)	0,25 µL
Primer Reverse (10 pmol/µL)	0,25 µL
DNA	(0,5-1,5) µL
H_20	qbp 12,5 µL
Total Volume	12,5 µl

B. Reaction Mix for Sequecing PCR

Reagents	Volume
BigDye®	0,5 µL
Buffer	0,5 µL
Primer M13F or Primer M13R (C = 3,2 pmol/µL)	0,5 µL
DNA product	3 µL
H_20	5,5 µL
Total volume	10 µL

8.3 Primers used in this study

Oligonucleotide primers (5'-3') and thermocycling conditions used for Polymerase Chain Reaction Amplification of the coding exons of the genes analyzed in this study. Note that all primers are tagged with an M13 universal primer, either forward (5'-3'): TGTAAAACGACGGCCAGT, or reverse (5'-3'): CAGGAAACAGCTATGACC. Universal M13 primers were used for sequencing.

Gene	Exon	Forward 5'-3'	Reverse 5'-3'	Tm
POLG1	2a	CTCCACGTCTTCCAGCCAGTAAAA	TTCTGCAGGTGCTCGACGCT	65
	2b	GGGCTGCACGAGCAAATCTT	AGCCCGTAACAGGACCTCAGAAAA	
	3	CAGTGGTTGTTGTGGAGTG	CAGCAAAGGCAACAGAGACA	
	4	GTCCACACCACCAAGCAGT	GTCCCAAGCACTATGCTCC	
	5-6	GCAGGAGCATAGTGCTTG	AAGCTAAGGTCCCCAACCTG	
	7	TCAGGGATTGGGCCAGTCTT	GAGCCAGGGGTCTTCTTTGT	
	8	GGCCTAGCCTTCGGCTTA	AGACAACCCCTACCCTACCC	
	9	GGGTGGGAGCAGATCTTATTG	TGAGAATGGAGCAAGGGTAGA	
	10	GGGACATTGTGAGAGAGAGA	ACTCTTTCCACTAGCCTGAG	
	11-12	CAGAGTGGGCATCTGGTAAT	AAGAGGAAGCCCTTTCCACC	
	13	ATGGCCCTTGCTGAATGCAG	TGGGCCTTGAGCAGAATGAG	
	14	GCAGGTACTCACGTTGGTTC	CTGTGGGAATCCAGGGTTAG	
	15-16	GGATGTGGGATAGATTCTGCT	CCTCAGAGCCCAGTTTCTACA	
	17-18	GTAGGAAGAGTCTCATTTGGG	CCTGGGTGTTAAAGTGGATG	
	19-20	TGAACATTCCTTGCCAAGGC	TCTGCCCATGCTCCAAAGGTA	
	21	GCTTCTACCCTGGAGTTAAT	CAAGGAACGCTCACCCAAAG	
	22	GTCATTGCTCCAGGAGTGAT	CTGTTCTCCAAGACCCACTT	
	23	GCTCCTTTGCTCACTTCTGA	CTACTGAAAAATGGCTGGCC	
C10orf2	1a	GGCACCTAAGGCATTTCAAG	ATGCAGAGAAAGTGGCCTGT	60
	1b	CACAGCTCAAAGGCCAGACT	CCAAGGGAAGACAAGACTGC	
	1c	GGTTACAGATGACACACTCAAGC	CTTCCCAGGACCGAAGGT	
	1d	CCCTGCCTTACTCCCTTACC	ACCCACTTGCTTTTGTCACC	
	2	GTCTTGGTTTCAAGGGTAGG	GATATGTCTGGGAAAGCAAGG	65
	3+4	GAGTTTTCAGGATGCTAGT	GTCACACCTACCCACTC	60
	5	CTTTCTGCTTTGCTCATGTCC	CCTTGCAGAGTTTTATGCTCC	65
TK2	1	TCCAAGTTATGGGTGCGTTC	CGGGAGTAGGTGGGCGCAT	60
	2	TTTTAGGCCAGGGAGTGAGCAT	TTCCTTCTCCCTGGAGATCCT	
	3	GCCCTTTCCGTAGCCATTAT	TTTCTCCGCTTCCTTCAAACC	

Gene	Exon	Forward 5'-3'	Reverse 5'-3'	Tm
TK2	4	TCACTTTCCCCTCAACACTTA	ACCATCATTCTCAGGTGC	60
	5	TGTCCTTCAGTGCCTTGTGA	AAGTTTCCCTTCCTGGCAA	
	6	ACATTTCTCAAGGCCTCCTG	CTCCATATCTGTCAATCGAATA	
	7	TCCAGTATTGCACGGATCAC	GGCAGACAGAATTCGGGAC	
	8	CTGTGTGCCTGCTTTGCTT	AGGTGGTTTCCCAGTTTGTC	
	9	CTCTGCTTGACACCCTTGG	CCCTCCCCTGTCTGCAAG	
	10	CTCCAGCTGTGAAAGGAGGAT	AAAATCAAGCTGGCCAGACA	
DGUOK	1	ATACCTAGGGCGGAAGTGCTCT	AGCTCCTTTCCCAGCCTCTGTC	60
	2	CTTGAGTTTGGGCGTTTGTG	AACTGGGCTACTTTACTCACTG	
	3	AAACCTGTTTGGGGAGGT	GATCAAACAGGCAGCACTGA	
	4	CTCGTGCCTTTCATTCCAT	GCTCTCTGTGCTGCAGGTAA	
	5	CCGAAGACTGCATTGTAGCA	GACATTTCCAACCATTTCCAG	
	6	TGGCGAGTATGTGAAACTT	CCATAGGCCTTGGAGTGTGA	
	7	CTCATGGGCTTGGCTGCATAT	GCTCTTGAGCGGCAGAGT	
MPV17	2	AATGTCAGGCGGCTTCTTA	AAGCCTCTGGCTTCCAAATC	65
	3	CCCTGGGGTTCAGAGTAC	CCTGCCCCAAGTATCAGAAT	
	4+5	TTCCCTTGGACTCTCACAC	ACCTGCACTTACCCCCTTTT	
	6+7	TGCAGGAATGTGCTCTTTG	GTTCCGTTTGGGGGTCTAAG	
	8	CTCCTTTGCCATTTCATGCT	CTTAAGCTCTGACCGGCAAC	55
RRM2B	2	GTAAGGATTCAACCCAGGCA	CCTGTAAAACAAGAAGGAACAC	65
	3	TCCCAAACACTTTGAGAAGAA	TTTGCTGGTCAAAGGATAAGC	
	4	TTAAGCGATAATGCTGATGTCC	TATTATGCTCAGAAACTTCCA	
	5	AGATCGTGCCACTGCACTC	CCATGCTGTAACTTTGATTCG	
	6	TCTTTGCAAACAGTTGTGGTAT	GTGACAGAGTGAGATCCTGT	
	7	GAACCACTTTATACAATGCTTG	CTGGTATTTTTATTGACCTACA	
	8	GAAAGTCCTCTTTCTATTTGGA	AACCTTGTTCATACAAAAC	
	9	CCTGACCTCTGTTTTGGTC	GCAAACCCCCAGTCCTTTA	
SUCLA2	1	GTCTAGCGGCTGCAGTGTT	CACACCTCACCCTTTCTCC	65
	2	GCAAGAGTGACTGCCAAATCA	TCCCACGTCACTGGCATTAT	60
	3	CTATGTCTGGCATGCTGCTC	TCCTCCAGTTGAGAAAAAGCA	65
	4	TGTGGATTTGCTTTTTCTCA	CATGCTCATGACATACAAACA	60
	5	CTGTGCTGGAGATGTGAG	GACAATTACTTCAGTTGTACGG	65
	6	TGGTAGCTGCTATTTCAGT	TGGGTCTAATAGTGTTTTGTT	60
	7	TCCCTAAATGAAAGACCATT	TGTATAAGCAACAAACACAGA	60
	8	TCTGTGTTTGTTGCTTATACA	TGATACTTCACATCCCTTAT	55
	9	GGGTATTGTGGTTTGGTCTGA	CTGAACTCCATGGAAATGTGA	60

Gene	Exon	Forward 5'-3'	Reverse 5'-3'	Tm
SUCLA2	10	AACCTGGGCTTTTACAGTG	TGCCTTAACATCTTTCAGCAA	65
	11	CCAGATGTTTTAAATGGAA	AAATGCAGTCCAAATCCTT	60
SUCLG1	1	TTTGTTCAGGCGACTGCT	GAAGCCGGAATCCCAAGC	65
	2	TTTTTGTGATCAAGTTGAATT	TTTTTCTGCAACAATCATGTG	60
	3	AGATTGCTTTTGCTTCTCTT	CCAAAGAATGCTCGCTCTTC	65
	4+5	TCCTTTTCTTACCCCAAGA	GGGTTTAAGGCAAATAAGC	
	6	TTAGTCACTCGAAGTGTTTGG	TCATCCAATGAAGACACCACA	
	7	TTGACCTAAATTCCATGGTTCA	GAAGGCCCTGCTCAGAAAG	
	8	GCCCAAGCCATTTACCATTT	GCTGCACATCAGAAAACC	
	9	GTGTTCAAACACCCTCATCC	TGTCAGGCACATAGGCTG	
TYMP	2+3	GGAGCACGTGGCTCAGAC	GGTAGGGTCTTGGAGGCTT	65
	4	AATCCAGCTGCACTCCTA	TGACCCTAATGATCCACCAG	
	5	GCTGCTAAGTCATTTACCA	GAGCCTGTGAACATGCAGA	
	6	GCTCCAGGTTCTCCATTGTC	GAGGGGTGAAGGGTAGGC	
	7+8	GCTGGAGTTCGACCAAGAAC	GGGAGAGGGGCTGAGAGG	
	9+10	CCCTGTGCTCGGGAA	GTCACGTGTTCATCGAG	
RARS2	1	GCTGCAGGGCAGAGGTTAG	CACCCTCCAGCTGACTGAG	62
	2	GGAATGCATTTGGAGCTCATT	CAGGAGTAATAGGCTGAGTAT	60
	3	CACCTTCTTCTGGTAAAGGTA	CAATGTCAGCTCAAGAGGAG	60
	4	GGGACCATGAAAGATGACTTA	GGTGGTACTCTCCAAGTTATG	60
	5	GTGGTGCTGGAGGTAGTGC	GATTGATTCACTGTGATTTCAAG	62
	6	GCCCTAATACCAAAGGCCAT	GGCAGGAGGCTCATATAAATT	60
	7	CTCCCTTTCCATAACACATGA	GTAGGACATACTACTTCATCC	60
	8	GACTTGTGTCAGATTATGGGG	GCAACAGAGCGAGACCCTG	62
	9	CACTCTGTAATGTACCCAACT	CACAAGCTTAACACGCAACTT	60
	10	CATGAGCCACCACACTCAG	GCTGCACATTGCATTCTGTTG	62
	11	GCTTGAATTGGCACTGCTCA	TGTCATCAGCTGTGGAATCC	60
	12	CTAAGTGTGGGTGGAATTCC	GAAGTTGCCCACTTTAAACATAC	62
	13	CTTAGACACTCTTCTTGTGGC	CCTTTCCACACCAGTATCTTG	62
	14	CTCTGCTCTGATGGTTAAAGT	GTGACACTTCAATGGAGGGA	60
	15+16	CTCATTCTGAATCTGTTCTTGAT	GCCTCTGGTCTTAGAATCAC	62
	17	CCTCCACTGTGTATGGATATT	GGCATACCTTTGGGAAAAGTC	60
	18	TTACAGACGTGAGACACTGC	CCAGTCTACAGTGACAGTAC	
	19	GGGCCAAGATCTAAAGCTTG	GTATGGCTTCAGATAAGGGG	
	20	GGCACTTTGACATATTGTTCC	CAGCAAGGCATCTCAGAATAG	
TRMU	1	ACAGCGCAGAAGAAGAGCAGT	ACTACACAGGTGGAGGGCGA	65

Gene	Exon	Forward 5'-3'	Reverse 5'-3'	Tm
	2	CTCAGGCACCAAGATGGAAAC	GAGGCCTCTTGCAGTCTTCAG	60
	3	CGTAGTGGCAGAGAATAACACCA	ACAGTTGTGACACCATCTCCAA	60
	4	CAGAGTGCTAGGATTACAGGC	GGCAGGGACACTGTTTACTAC	65
TRMU	5	GAGTGTTGATGTCTGCCTCTGA	CCTCAGCAAACTCCTCCATCT	65
	6+7	TCTAAGGCTCTGGCATCGTGT	GGACGACAGGAACTCTGGTCTAG	60
	8	GATGTGCTCAGGTGCTTGGT	GACCAGCATACAACTCAGCCTA	65
	9+10	GTGTGCTGGTAGGACAGTTGTT	GTGACACCAGAGTGGAAATCC	65
	11	TCTCCTGTTCAGCAGCAGCA	ACACAGGTCAGCATCGCAGG	65
	1+2	AGCAGGTGGCACCATACTC	TGCCATTATGGTCACTGAAG	65
	3	TGGTAGACTGCCAGTAAATGATAG	TCTTCAAGAACAGGCCC	60
TSFM	4	TTTCCGTTGAGTCTGTAGCTTG	AGCATTTAAGCAGAGATGTGAAG	60
	5	ACGCCTGGCCAATACTTTTC	CACACCACTTCTCACGTTGC	65
	6	GACTGTCTTCCAAACTGGGC	CTCGGTCTGAAGAGGTTTGG	65
	1+2	AGCTCTAACTTCCGCCGGAA	TCCAGGTCCCATCAGTAGATA	60
	3	TGCCTCTAGCACTGGAACTT	TCCTGACAAGAGGCAGCTTCT	65
TUFM	4+5	GTGAACTGAAGCGCTCGTTG	AGACACAAAGCAGAGCTCTG	60
	6+7	CAGAGCTCTGCTTTGTGTCT	AGAGGGAAGGCACAAGGGA	65
	8+9	TCCCTTGTGCCTTCCCTCT	GGAACTATGAGTGAAGCAAAGG	65
	10	TTAAGGAATGAAGGCACCCTGG	TCCTCCCCTATCCTCTCCAAT	65
	1	GCTCTTACAACATTGGCTGC	GTGTCACGGCCTTCCATTG	65
	2	GGAGGAATAATGTCCACCATAC	TGTCAAAAGTCCGCTGTTACC	65
GFM1	3	CTGGTCTCAAACTCTTGGGC	GAAGTGCTCTAGGTCCTAAGTTAGTC	60
	4	GCACCTAAGGGTTGGTGAATAG	GCCAATTTACTAAAATCAGTGTTCC	
	5	TGTGACCTGATGGTAACACCC	TCTGCGTTTGATGAACTTATCC	65
	6	CTTGACCTTAAACTTGCCTATTTC	GCATCATGTCCCCTCCC	
Gene	**Exon**	**Forward 5'-3'**	**Reverse 5'-3'**	**Tm**
	7	AGTAAATCACTTCCCAGCCC	AACAAATCATACTTCCAACTCAAAAC	
	8	CAGCAGTAATATCCCACACACG	GCTTTGGAAATTCTCTACCTGC	
	9	GGAGGGAGTTATGAAGCTTTCC	CTAGTGGGGCTCAGGATACC	
	10	AGCAGAGAGATTCTGCCACG	CTTGATGAGTGTCCTACTCAACG	
GFM1	11	TTATTTTGGGAAGGAGGAACC	AAGAAGATCTCAATACCAACAGAGG	65
	12	TTGGCTGTGTGGTATCCAAG	AACCAGAGTGACGCATGAAG	
	13	TCAACAATGGTGACGTTTTAGTG	GGCCAAATAATCCCAAAGTG	
	14	TTAATAGGAGTTATTTTATTTT	TGATAGACAACATGATGGAAGA	
	15	GAGGGGTGAAATACAGCACAC	GCCTAGTGCTCTGGGTGAAG	
	16	TTGCCGTTTGTGTTTGTACC	AAGCCACAAATTCAGCAAGG	

GFM1	17+18	AAATGGCTAAAATGCGTCTG	GAATTTCTTGTTTCAGCAGCC	65
TAZ	1	TCAGGGGCCAGTGTCT	CTCGTACAGCACCTCCCT	65
	2	GGAGGTGGGACTTA	GGAGGTGGGACTTA	55
	3+4	CACTTTGGGCTGTAGGGAAA	CCATAGGTCCCTCCAAAACA	65
	5	AGGCTTGTGGGGTGA	AAACTCCTGGGCTTGAGT	60
	6+7	TTTGGAGAGTGTTGAGG	CCATCTACCCCCATACAA	65
	8+9	GCTGGCACAGAAGCT	TGGCCTCCATAGTGCTGA	65
	10+11	GCTGTGGGCACTCCTA	GAGGGCACTTGAGAGGGTCT	65
TTC19	1	CGAACAAGAGGTTTAGAGAACCA	AGCTCAGGAGCCGGAACAT	60
	2	GATGTCCTGCTCAGTCGTCA	GAGAGAGTAATGCGCAGCAA	
	3	GCTGACCTCCCTGTCATCTC	GAACATCGTGAAAGTCAGGAAT	
	4	TGATTGCTGTCCTGTGCTTC	TGCCTATGTCTGTCTGCCTTC	
	5+6	TGTTGCATCAGACTGGCACT	TCCCAGAGTCAAAGTTTCCTG	
	7	CGGAGGTGGAGGTTGCTCT	TTCCAAAACTTCTGCTGATACC	
	8	AGTGACATGTGGGTCCCATT	CCAAGGAGGATCTAGGACCA	
	9	TCCCTCATCTTTTGTGGAATG	CCCTTGCCCTCCCTACATAC	
	10	TGCATTCATGCTCTCTCTTCA	TGCATTCATGCTCTCTCTTCA	

Legend: Tm – Temperature of melting in ºC

8.4 SDS and BN-PAGE experiments

A. BN-PAGE gel of 5-13%

	Gradient gel 5%	**Gradient gel 13%**	**Stacking gel 5%**
Acrylamide 40% (29:1)	0,4 ml	1 ml	0,5 ml
BN-gel buffer 3x (1,5M aminocaproic acid; 150 mM Bis-Tris, pH 7,0)	1,33 ml	1,33 ml	1,67 ml
Glycerol		0,8 ml	
H_2O	2,27 ml	0,87 ml	2,83
APS 10%	44 µl	12 µl	55 µl
TEMED	4,4 µl	1,2 µl	5,5 µl
Total volume	4 ml	4 ml	5 ml

B. SDS-PAGE gel of 12%

	Resolving gel 12%	Stacking gel 4%
Acrylamide 30%	4 ml	1,33 ml
Tris 1,5M, pH 8,8	2,5 ml	-
Tris 0,5M, pH 6,8	-	2,5 ml
H_2O	3,35 ml	6 ml
SDS10%	100 µl	100 µl
APS 10%	50 µl	60 µl
TEMED	5 µl	7 µl
Total volume	10 ml	10 ml

8.5 Papers published

Topical Review Article

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Infantile-Onset Disorders of Mitochondrial Replication and Protein Synthesis

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Abstract

Most inherited mitochondrial diseases in infants result from mutations in nuclear genes encoding proteins with specific functions targeted to the mitochondria rather than primary mutations in the mitochondrial DNA (mtDNA) itself. In the past decade, a growing number of syndromes associated with dysfunction resulting from tissue-specific depletion of mtDNA have been reported in infants. MtDNA depletion syndrome is transmitted as an autosomal recessive trait and causes respiratory chain dysfunction with prominent neurological, muscular, and hepatic involvement. Mendelian diseases characterized by defective mitochondrial protein synthesis and combined respiratory chain defects have also been described in infants and are associated with mutations in nuclear genes that encode components of the translational machinery. In the present work, we reviewed current knowledge of clinical phenotypes, their relative frequency, spectrum of mutations, and possible pathogenic mechanisms responsible for infantile disorders of oxidative metabolism involved in correct mtDNA maintenance and protein production.

Keywords

mtDNA, OXPHOS, DNA replication, mitochondrial DNA depletion syndrome, protein synthesis

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Mitochondria play an important role in a number of cellular processes. Their primary function is to supply most of the cellular adenosine triphosphate (ATP) requirements through oxidative phosphorylation (OXPHOS), a process that involves 5 protein complexes located along the inner mitochondrial membrane. Biogenesis of mitochondria proceeds in 2 main steps: (1) formation of a mitochondrial mass, depending mainly on nuclear transcripts of mitochondrial proteins; and (2) energetic differentiation, which can be hampered by the depletion of mitochondrial DNA (mtDNA).[1] Thirteen of the key structural polypeptides that constitute the multimeric subunits of the respiratory chain complexes are mtDNA encoded, in addition to 2 ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) that are required for initiating translation and protein synthesis.[2] Approximately 90 of the remaining proteins that make up the respiratory chain complexes are encoded by nuclear DNA (nDNA). Therefore, although human mtDNA encodes the basic machinery for protein synthesis, it depends entirely on the nucleus for the provision of enzymes for replication, repair, transcription, and translation. This dependency lies at the heart of several newly recognized human diseases that are characterized by secondary abnormalities of mtDNA.

A genetic classification of mitochondrial diseases of oxidative metabolism distinguishes 2 principal categories: disorders owing to mutations in the mtDNA, which are governed by the laxer rules of mitochondrial genetics, and disorders owing to mutations in nDNA, which are ruled by Mendelian genetics. Moreover, the crosstalk between the 2 genomes is crucial for the cellular regulation of mtDNA integrity and copy number and correct mitochondrial protein production. As all of the factors are nDNA encoded, it is not surprising that mutations in genes involved in mitochondrial replication and maintenance can disrupt the integrity of the "tiny" mitochondrial genome.[3] Maintenance of mtDNA is controlled by an intricate homeostatic network, whose effectors are the various components of the mitochondrial "replicosome" and the many enzymes and carrier proteins that provide the mitochondrion with a balanced supply of deoxyribonucleotides. In principle, abnormalities in each of those proteins can cause intergenomic miscommunication, a third category in the genetic classification of diseases of oxidative metabolism. There are different genes

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that have a crucial role in these pathways, including: (1) genes such as *POLG1* and *C10orf2/PEO1 (Twinkle)*, whose products function directly at the mtDNA replication fork; (2) those involved in the salvage pathways of mitochondrial deoxynucleotides or in the control of the mitochondrial deoxynucleotide triphosphate pools, including *TK2, DGUOK*, and *MPV17*[4]; and (3) genes influencing the several steps of mitochondrial protein replication, such as those encoding elongation factors, aminoacyl-tRNA synthetases, or even mtDNA-encoded tRNA. A mutation in any of these genes produces severe biochemical defects of OXPHOS or is harmful for mtDNA maintenance, or both. As a consequence, patients—especially infants and children—suffer from severe neurological illness, because the developing brain is foremost in requiring sufficient oxidative substrates for its function.

Most inherited mitochondrial diseases in infants result from mutations in nuclear genes encoding proteins with specific functions targeted to the mitochondria rather than primary mutations in the mitochondrial genome itself. There are a number of excellent reviews that have dissected the role of mtDNA in human pathologies at any age, and particularly in infancy.[5-7] In the present work, we provide a molecular compendium of the clinical presentations of oxidative metabolism disorders in genes determining, or contributing to, correct mtDNA maintenance and protein production. The list of clinical phenotypes will be accompanied by their spectrum of mutations and possible pathogenic mechanisms, which would be useful for general child neurologists when facing similar cases.

Etiologies and Clinical Correlates

Disorders of mtDNA Intergenomic Miscommunication

The mitochondrial and nuclear genomes exert a dual genetic control on oxidative metabolism and associated systems. Abnormalities in components involved in mtDNA transcription/replication can cause intergenomic miscommunication disorders. In these disorders, a primary nuclear gene defect causes secondary mtDNA loss or deletion, which leads to tissue dysfunction.[8]

Defects of POLG1. The *POLG1* gene (OMIM 174763), encoding the catalytic subunit of DNA polymerase gamma (pol γ), is located on chromosome 15q25 and represents the most common nuclear gene causing mitochondrial disorders, and there are about 150 mutations that have been described (see http://tools.niehs.nih.gov/polg for the complete mutation database). The enzyme pol γ is the single mitochondrial DNA polymerase and is made up of an "exonuclease domain," located on the N-terminus with predominant proofreading function, and a "polymerase domain," located on the C-terminus, which is mostly in charge of mtDNA replication. Because of its essential role for mtDNA replication and repair, it is not surprising that pol γ-related syndromes are associated with mtDNA deletions or depletion in tissues.[9]

Diseases resulting from mutations in *POLG1* are associated with an extremely heterogeneous spectrum of clinical presentations, ranging from autosomal dominantly and recessively inherited forms of progressive external ophthalmoplegia, to juvenile spinocerebellar ataxia and epilepsy with or without dysarthria, to Alpers-Huttenlocher syndrome, probably the earliest description of a mitochondrial disorder.[7,10]

Approximately 45 different point mutations (Figure 1A) in *POLG1* cause Alpers-Huttenlocher syndrome, an early onset, autosomal recessive mtDNA depletion disorder characterized by psychomotor retardation, continuous seizure activity (*epilepsia partialis continua*), and liver failure in infants and young children.[11,12] Brain magnetic resonance imaging includes signal abnormalities in the basal ganglia and thalami, irregularly widened ventricles, and partial pachygyria. Patients usually, but not always, present multiple deficiencies of the respiratory chain enzyme complexes and mtDNA depletion in the liver, but both may be normal in skeletal muscle. The clinical picture overlaps with hepatocerebral syndrome, which is probably among the most severe diagnoses of infancy.[10]

Severe heterozygous mutations of *POLG1*, often affecting the "DNA-binding" and the "catalytic" polymerase domains of the enzyme, cause mtDNA depletion and manifest in infancy or early childhood with severe liver and brain disease.[13] The 2 most common *POLG1* mutations detected in Alpers-Huttenlocher syndrome are A467T and W748S, and they can be either homozygous or heterozygous in combination with other mutations. Carrier frequency for these mutations is higher in Western countries: 1:125 for W748S in Finland; 1:50, combined for both variants, in Norway; 0.6% of the population in Belgium for A467T; and single ancestral founder mutations are hypothesized for both variants.

Defects of C10Orf2 (Twinkle). The mitochondrial protein Twinkle, encoded by PEO1/C10Orf2 (OMIM 606075) on chromosome 10q24, is an mtDNA replicative helicase, active as a homohexamer and bound to mtDNA in mitochondrial nucleoids (Figure 2).[14] Mutations in C10Orf2 cause dominant disorders such as pure, adult-onset external ophthalmoplegia associated with multiple mtDNA deletions, or recessive clinical conditions including severe neonatal/infantile-onset hepatoencephalopathy or infantile-onset spinocerebellar ataxia associated with mtDNA depletion in the brain and liver, but not in skeletal muscle.[13]

Spinocerebellar ataxia of infancy is a severe autosomal recessively inherited neurodegenerative disorder that manifests after 9-18 months of age by progressive atrophy of the cerebellum, brain stem, and spinal cord; ataxia during the first 2 years; hypotonia and sensory axonal neuropathy; optic atrophy; hearing impairment; and ophthalmoplegia.[13] Patients usually survive to adulthood. The severe neurological phenotype observed in this condition and the absence of muscle involvement suggest that Twinkle may play a crucial role in the maintenance and function of specific affected neuronal subpopulations.[15]

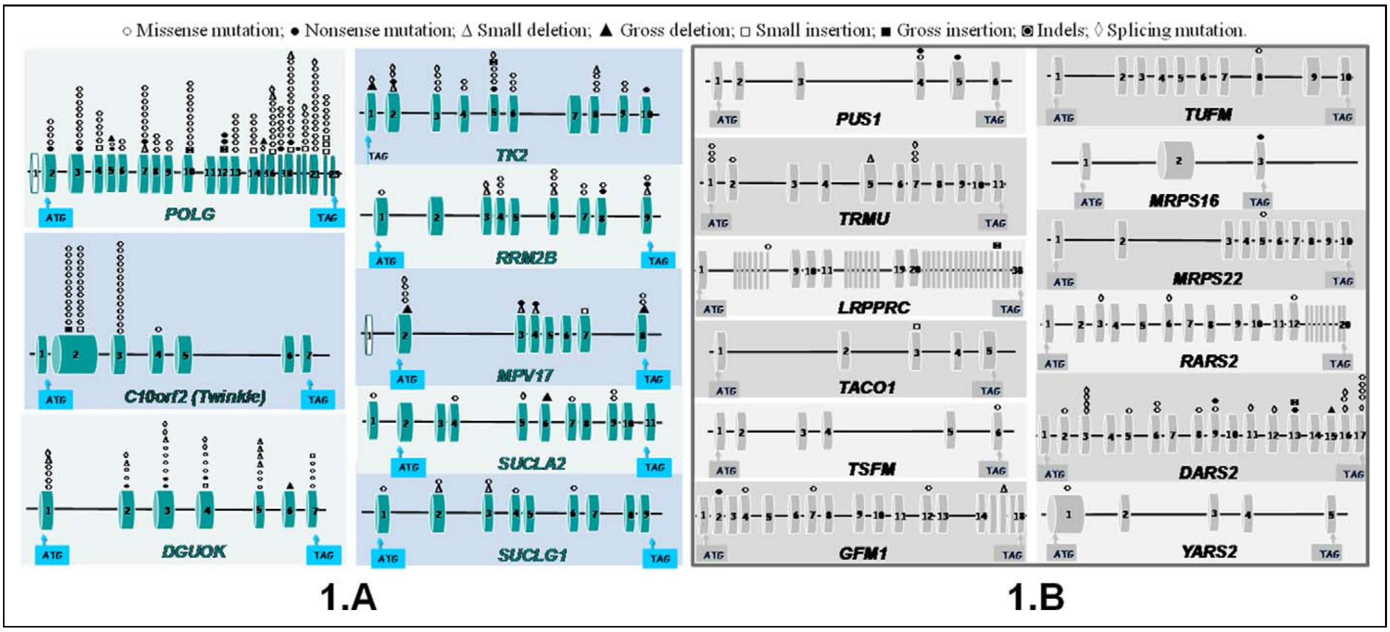

Figure 1. Morbidity map of the several genes associated with (A) mitochondrial depletion syndrome and (B) disorders of mitochondrial protein synthesis. Data are taken from the HGMD Professional database (www.hgmd.cf.ac.uk/; latest accession date June 12, 2010).
Empty (○) and filled (●) circles indicate missense and nonsense mutations, respectively, whereas empty (Δ) and filled (▲) triangles designate small- and large-scale deletions, respectively. In addition, empty (□) and filled (■) squares indicate small and gross insertions, whereas scattered squares (▣) and diamonds (◇) show insertion/deletion mutations or gene variants occurring at splice site sequences, respectively.

Spinocerebellar ataxia of infancy represents the second most common heritable ataxia in Finland because of a founder mutation (Y508C) with a carrier frequency of about 1:200. The Y508C mutation has also been described in compound heterozygosity with A318T in patients with severe early onset encephalopathy, signs of liver involvement, and mtDNA depletion in the liver. The hepatocerebral form of mtDNA depletion syndromes can also be caused by a different recessive mutation in Twinkle (Figure 1A).[16]

Defects of TK2. Thymidine kinase (TK2) is an intramitochondrial pyrimidine nucleoside kinase that phosphorylates deoxythymidine, deoxycytidine, and deoxyuridine (dNTP), thereby participating in the salvage pathway of deoxynucleotide synthesis in the mitochondria.[17] Mitochondrial dNTP pools arise either through active transport of cytosolic dNTP or through salvage pathways by the action of 2 mitochondrial deoxyribonucleoside kinases, TK2 and deoxyguanosine kinase, responsible for the salvage pathways of pyrimidine and purine nucleotides, respectively (Figure 2). Both pathways are essential for the replication of mtDNA, since the mitochondrion is unable to synthesize deoxynucleotides *de novo*. In addition, since mtDNA is replicated throughout the whole cell cycle, there is a constant need of nucleotides for mtDNA replication.[18] In nonreplicating tissues, *TK2* is one of the indispensable enzymes for mtDNA maintenance.[17]

Mutations in the *TK2* gene (OMIM 188250) on chromosome 16q22 affect primarily muscle tissue, with little or no effect on the liver, brain, heart, or skin.[4] The typical manifestation of *TK2* mutations is a severe, rapidly progressing myopathy of infantile or childhood onset. Electromyography is myopathic, creatin kinase is elevated to several thousand (> 1000 U/L), and lactate is usually mildly elevated (< 2× normal values). Morphologically, muscle biopsy in *TK2* patients may show dystrophic features with cytochrome *c* oxidase-negative fibers and ragged-red fibers.

The first children described with *TK2* mutations had severe infantile myopathy with motor regression and early death from respiratory insufficiency.[17] Mutations in *TK2* seem to be specifically associated with the myopathic form of mtDNA depletion, although a spinal muscular atrophy-like phenotype has occasionally been reported.[19] The disease course is rapidly progressive, leading to respiratory failure and death in months or years, but milder phenotypes with slower progression and longer survival have been reported.[13]

Approximately 25 different pathogenic mutations in *TK2* have been published so far (Figure 1A), either as recessive homozygous or compound heterozygous mutations, and phenotypes may be explained by variable degrees of residual activity of the mutant enzymes. The prevalence of *TK2* mutations among myopathic forms of mtDNA depletion is thought to be approximately 20%. None of the reported patients displayed homozygous loss-of-function variant, suggesting that some residual enzymatic activity is mandatory to avoid embryo lethality. Accordingly, knocking out in mice leads to high embryonic lethality.[20]

Defects of DGUOK. Deoxyguanosine kinase (OMIM 601465) is a 2-deoxyribonucleoside enzyme that catalyzes the first step of the mitochondrial deoxypurine salvage pathway, the

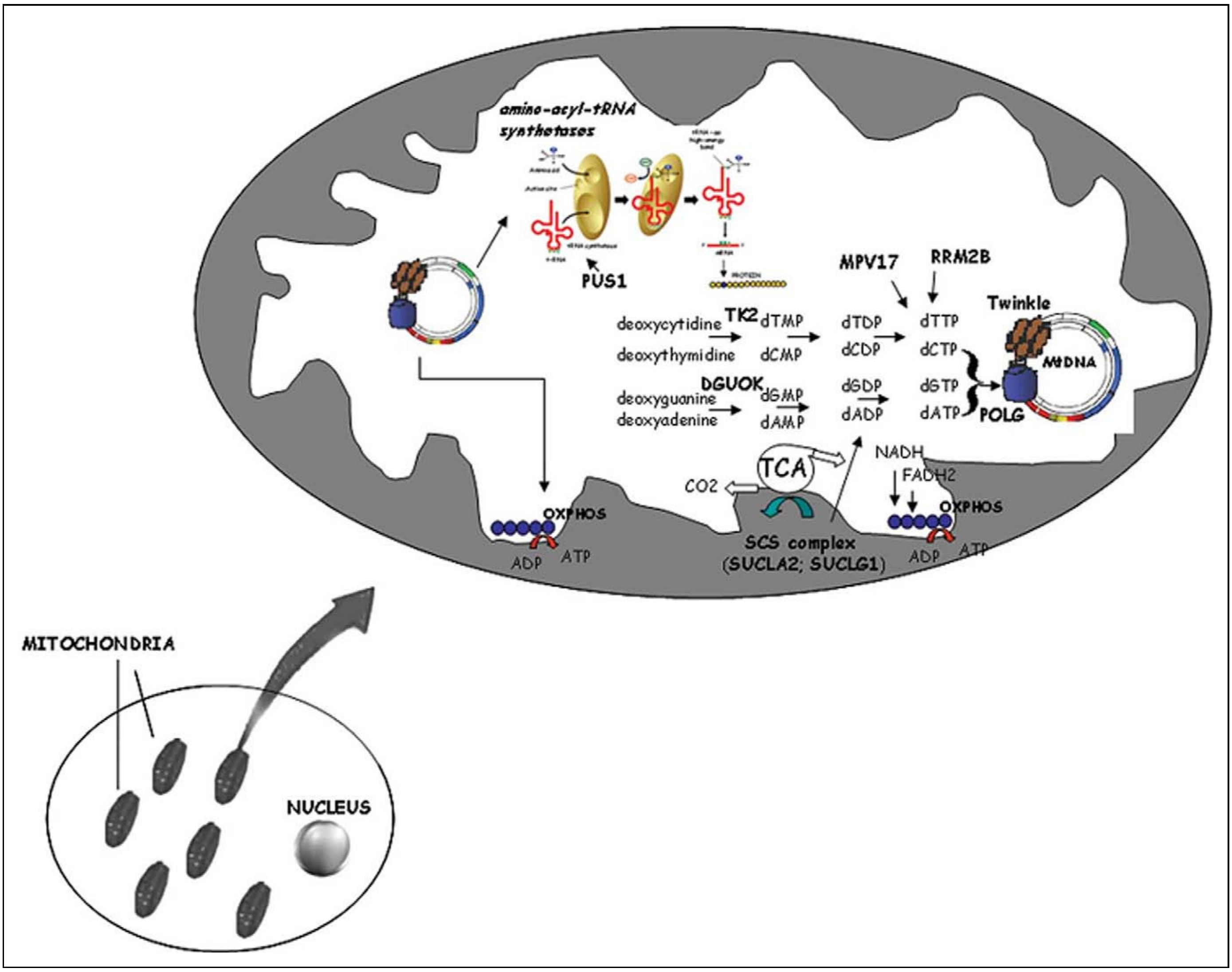

Figure 2. Schematic overview of the mitochondrion and the mitochondrial disease genes involved in diseases of mitochondrial protein synthesis and mtDNA depletion syndromes. Zooming in on the mitochondrion allows identification of genes (namely, *POLG1* and *Twinkle*) thought to be involved in replication of mitochondrial DNA (mtDNA); those assumed to affect the metabolism of the mitochondrial deoxynucleotide (dNTP) pool (via progressive phosphorylations of deoxythymidine, deoxycytidine, deoxytadenine, and deoxiguanosine); and those belonging to the tricarboxylic acid cycle and affecting the respiratory chain complexes (OXPHOS). Moreover, the supposed role of genes involved in the complex machinery of mitochondrial protein synthesis (including the aminoacyl-tRNA synthetases) is illustrated.

phosphorylation of purine deoxyribonucleosides into the corresponding nucleotides deoxyguanosine and deoxyadenosine necessary for the maintenance of mitochondrial deoxynucleotide pools (Figure 2).[4,15] The typical phenotype of mutations in the *DGUOK* gene on chromosome 2p13 is characterized by neonatal onset of progressive liver disease and feeding difficulties, usually with neurological dysfunction (hypotonia, nystagmus, and psychomotor retardation), by the age of 3 months. Peripheral neuropathy and renal tubulopathy have occasionally been reported.[21] All patients often have elevated levels of tyrosine and phenylalanine in their plasma as an unspecific marker of liver dysfunction, and hyperlactatemia is always manifested. Magnetic resonance spectroscopy can reveal a lactate peak in the brain.[22] Depletion of mtDNA has been documented only in the liver and results in combined respiratory chain deficiencies in the liver, whereas the amount of mtDNA is usually normal in muscle and fibroblasts. Histological analyses of the liver biopsy show variable findings, typically microvacuolar steatosis, cholestasis, fibrosis, and cirrhosis. In most cases, there is a rapidly progressive liver disease and neurological deterioration, with death occurring by the age of 12 months or shortly thereafter.[22] No brain tissue has been investigated in cases of *DGUOK* deficiency.

More than 80 affected patients from approximately 50 families have been reported, and over 40 different *DGUOK* mutations have been identified (Figure 1A).[13] The infantile hepatocerebral form of mtDNA depletion is the almost invariable clinical presentation. Overall, variants in *DGUOK* account for approximately 10%-15% of the cases of mtDNA depletion.[13,22,23] Genotype-phenotype correlation studies show that patients who harbor null mutations usually have early onset liver failure and significant neurological disease, including hypotonia, nystagmus, and psychomotor retardation, and death before 2 years of age. Patients carrying missense mutations usually have isolated liver disease, a better prognosis, and longer survival. Although orthotopic liver transplantation is considered necessary to avoid an inexorable liver failure, the presence of significant neurological signs indicates multi-organ involvement, which precludes *ipso facto* this possibility.[24]

Defects of RRM2B. The *RRM2B* gene on chromosome 8q23 encodes the small subunit of p53-inducible ribonucleotide reductase, a heterotetrameric enzyme responsible for *de novo* conversion of ribonucleoside diphosphates into the corresponding deoxyribonucleoside diphosphates that are crucial for DNA synthesis.[25] The enzyme is the main regulator of the nucleotide pools in the cytoplasm, and its small subunit is expressed in postmitotic cells, where it probably has a key function in maintaining the mitochondrial deoxynucleotide pool for mtDNA synthesis (Figure 2).

Mutations in *RRM2B* (Figure 1A) usually result in hypotonia, lactic acidosis, failure to thrive, and tubulopathy in the first months of life. The disease has a rapid progression and leads to death in a few months. Fifteen patients with this phenotype have been reported so far. The associated complex phenotype suggests that the consequences of a defective mitochondrial deoxynucleotide pool can vary dramatically depending on the residual amount of the functional enzyme. Such variability has not been observed in other multiple deficiencies of oxidative metabolism caused by a faulty deoxynucleotide pool.[13] Recently, it has been shown that inactivating mutations in *RRM2B* also cause severe neonatal or infantile forms of mtDNA depletion, with profound reduction of mtDNA copy numbers in skeletal muscle.[25]

Defects of MPV17. The *MPV17* gene is located on chromosome 2p23-p21 and encodes a mitochondrial inner membrane protein of unknown function recently recognized as responsible for mtDNA depletion. The clinical presentation is that of severe liver failure, hypoglycemia, growth retardation, neurological symptoms, and multiple brain lesions during the first year of life.[26] Marked mtDNA depletion in the liver is the molecular hallmark associated with multiple defects of respiratory chain complexes. Normal or mildly reduced levels of both mtDNA content and respiratory chain enzyme activities were also found in muscle.[18] Histological analyses of the liver have revealed swollen granular hepatocytes, microvesicular steatosis, and focal pericellular and periportal fibrosis.

About 20 different mutations have been reported in infantile-onset hepatocerebral syndrome and in Navajo neurohepatopathy (Figure 1.A), which is an autosomal recessive multisystem disorder found in the Navajo of the southwestern United States.[4] Three main subtypes are to be considered: infantile-onset (before 6 months) and childhood-onset (before 5 years) forms with hypoglycemic episodes and severe progressive liver dysfunction requiring liver transplant, and a "classic" form with moderate hepatopathy and progressive sensorimotor axonal neuropathy. The 3 forms are also associated with variable degrees of demyelination in both the central and the peripheral nervous system. Remarkably, the same pathogenic mutation (R50Q) detected in an Italian kindred with hepatocerebral syndrome was later found to be responsible for Navajo neurohepatopathy, raising the possibility of an unexpected ancient relationship, or a mutational hot spot.[15]

Defects of SUCLA2 and SUCLG1. Succinyl CoA synthase is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinate and adenosine triphosphate (ATP) or guanosine triphosphate (GTP) from succinyl-CoA and adenosine diphosphate (ADP) in the tricarboxylic acid cycle (Figure 2). This enzyme is made up of 2 subunits, α and β, encoded by *SUCLG1* on chromosome 2p11 and *SUCLA2* on 13q12, respectively. Mutations in *SUCLA2* and *SUCLG1* cause an encephalomyopathic form of infantile mtDNA depletion syndrome, but *SUCLG1* can also cause a very severe disorder with antenatal dysmorphisms, neonatal metabolic crisis, and early death, probably depending on the lower residual amount of the protein.[27,28] A useful diagnostic clue in Succinyl CoA synthase disorders of succinyl CoA synthase is a "mildly" elevated urinary methylmalonic acid, which is detected in all patients, and presence of tricarboxylic acid cycle intermediates (methylcitrate, lactate, carnitine esters, 3-hydroxyisovaleric-acid) in most cases. Some patients die as infants (sudden infant death syndrome), but some of them have a longer survival.

The clinical features of patients with mutations in *SUCLA2* include early childhood hypotonia, developmental delay, and almost invariably, progressive dystonia and sensorineural deafness. MtDNA depletion can be detected in muscle, and enzyme analysis shows combined deficiencies of the activities of oxidative metabolism. Nearly 30 patients have been associated with mutations in *SUCLA2* (Figure 1A); a great majority of them are from southern Italy and the Faroe Islands, where a founder mutation has been recognized, with a predicted carrier frequency of 1:25.[27] Mutations in *SUCLG1* have thus far been reported in only a handful of families (Figure 1A).

SUCLA2 and SUCLG1 mutations seem to disrupt an association between succinyl CoA synthase and mitochondrial nucleoside diphosphate kinase, resulting in an unbalanced mitochondrial dNTP pool and eventually, mtDNA depletion (Figure 2). Moreover, the mtDNA depletion is frequently mild to moderate in skeletal muscle. The modesty of this depletion, along with the methylmalonic aciduria, suggests that Succinyl CoA synthase deficiency may also impair at least 1 collateral pathway that plays a role in the development of the disease.[10]

Disorders of Mitochondrial Protein Synthesis

Mitochondria contain a separate protein-synthesis machinery to produce the polypeptides encoded in mtDNA, and many mtDNA disease mutations affect this machinery. In humans, the mitochondrial rRNA and tRNA are encoded by mtDNA, whereas all proteins involved in mitochondrial translation (ie, aminoacyl-tRNA synthetases; initiation, elongation, and termination factors) are encoded by nuclear genes.[29] In general, when multiple enzymes of oxidative metabolism are affected, the genetic defect is presumed to be located in genes needed for mtDNA maintenance, mitochondrial transcription, and translation, including post-transcriptional or post-translational processes, import of nDNA-encoded proteins into the mitochondrion, and mitochondrial membrane biogenesis.[30] One category of mitochondrial disorders that is becoming increasingly diagnosed is that of nuclear gene mutations that cause mitochondrial protein synthesis disorders without evidence of mtDNA involvement. This group of disorders is highly heterogeneous and usually shares a combined disorder of respiratory chain complexes. To date, the underlying genetic defects encode mt-tRNA modifying and aminoacylating enzymes (*PUS1, TRMU, DARS2, RARS2*, and *YARS2*), a stability factor and translational activator (*LRPPRC, TACO1*, respectively) for mRNAs, translation factors (*GFM1, TUFM, TSFM*, and *C12orf65*), components of the mitochondrial ribosome (*MRPS16/22*), and factors that mediate mitochondrial ribosome assembly (*SPG7, AFG3L2*). Clinical presentations are diverse, but other than the mt-tRNA modifying defects, almost all patients have neurological deficits. In childhood, mutations in aminoacyl-tRNA synthetases are becoming particularly frequent, and the other etiologies are less common. Mutations in *SPG7* and *AFG3L2* occur mostly in adult-onset spinocerebellar ataxia or spastic paraplegia.

Heteroplasmic mtDNA deletion syndromes should not be included in their strict sense in this category of disorders, but for completeness, it must be said that they invariably result in severe defects of mitochondrial protein synthesis.[4,7] Moreover, when clinically manifesting as the Pearson bone marrow-pancreas syndrome (OMIM 557000), they represent an important etiology of infantile sideroblastic anemia, with vacuolization of marrow precursors and exocrine pancreatic dysfunction. In Pearson syndrome, severe, transfusion-dependent macrocytic anemia usually starts in infancy and is accompanied by pancytopenia and variable hepatic, renal, and endocrine failure. Death often occurs in infancy or early childhood as result of infection or metabolic crisis.[31] Eventually, patients may recover from the refractory anemia, and older survivors develop Kearns-Sayre syndrome (OMIM 530000), which is a mitochondriopathy characterized by progressive external ophthalmoplegia, pigmentary retinopathy, and weakness of limb skeletal muscles.

Defects of PUS1. The Pseudouridine synthase 1 (*PUS1*) gene on chromosome 12q24 encodes an enzyme that converts uridine into pseudouridine at several cytoplasmic and mitochondrial tRNA positions and thereby improves translation efficiency in the cytosol as well as the mitochondrion. Thus, PUS1 is not part of the translation machinery, but it is required for protein synthesis because of its function in post-transcriptional modification of tRNA. Pseudouridylation is the most frequent modification in tRNA, and it appears to stabilize both base pairing in stems and base stacking in the anticodon loop. Also, it is thought that the gene product may affect the interaction of the tRNA with its cognate aminoacyl tRNA synthetase (Figure 2).[29]

Mutations in *PUS1* (Figure 1B) are responsible for the rare myopathy, lactic acidosis, and sideroblastic anemia syndrome (OMIM 600462). Severity of symptoms varies between and within affected families and sometimes includes mental retardation. Mutations result in decreased pseudouridylation of some cytoplasmic and mitochondrial tRNA and ultimately affect protein translation.

Defects of TRMU. The *TRMU* gene on chromosome 22q13 encodes an evolutionarily conserved protein involved in mitochondrial tRNA modification and is important for mitochondrial translation. Defects in tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (TRMU), a mitochondria-specific enzyme that is required for the 2-thiolation on the wobble position of the tRNA anticodon, result in reduced steady-state levels of 3 tRNA (tRNALys, tRNAGln, and tRNAGlu) and consequently, impaired mitochondrial protein synthesis.[29,32] Recently, mutations in *TRMU* (Figure 1B) were detected in patients with acute liver failure in infancy.[32] All patients harboring mutations in this gene showed combined deficiencies of oxidative metabolism, with (near) normal complex II activity, and a clear defect in mitochondrial translation. Interestingly, it has been hypothesized that human *TRMU* may modulate the phenotypic manifestations of deafness-associated mtDNA mutations.[33]

Defects of LRPPRC. The *LRPPRC* gene is located on chromosome 2p21. Mutations in *LRPPRC* (Figure 1.B) lead to the French-Canadian subtype of Leigh syndrome, associated with a profound deficiency of complex IV of the OXPHOS system.[34] Patients exhibit neonatal or infantile onset hypotonia and psychomotor delay, and bilateral hyperluciencies of basal ganglia, like other more common forms of Leigh syndrome.[7] Leucine-rich PPR-motif containing protein (also known as LRP130) is a member of the pentatricopeptide family, acts as potential translational activator, and plays a role in the translation and stability of mRNA of subunits I and III of cytochrome *c* oxidase.[35] Leucine-rich PPR-motif containing protein has been suggested to function together with heterogeneous nuclear ribonucleoprotein K and RNA polymerase in coupling the mitochondrial transcription and translation machineries.[36] This manner is analogous to the yeast system. Moreover, *LRPPRC* binds not only mitochondrial but also nuclear mRNA, indicating that it could be involved in coordinating nuclear and mitochondrial gene expression.[37]

Defects of TACO1. Upon genome-wide linkage analysis and chromosome transfer in a Kurdish patient presenting with Leigh syndrome combined with isolated cytochrome *c* oxidase deficiency, it was identified as a homozygous single base insertion (Figure 1B) in the *CCDC44* gene (later renamed *TACO1*) on chromosome 17q.[6,38] It is noteworthy that *TACO1* represents the first specific mammalian mitochondrial translational activator, opening the possibility to a new class of proteins controlling efficiency of mitochondrial translation.

The clinical features of the original Kurdish kindred have recently been reviewed.[39] In 5 children, mutant *TACO1* resulted in a relatively late-onset Leigh syndrome (onset range 4-13 years) characterized by short stature, mental retardation with autistic-like features, and a slowly progressive array of motor symptoms related mainly to basal ganglia involvement.[39] This less aggressive clinical progression is unusual if one also considers the severity of the associated cytochrome *c* oxidase deficiency in tissues from the patients.

Experimental work in patients' cultured fibroblasts suggested that *TACO1* is necessary for the rate of synthesis of full length subunit I of cytochrome *c* oxidase. The mutation would result in premature protein truncation and likely compromise assembly and activity of cytochrome *c* oxidase, complex IV of the mitochondrial respiratory chain. Remarkably, deletion of YGR02, the *TACO1* yeast ortholog, did not show any translation defects and did not hamper the activity of complex IV.[38] Unlike the human counterpart, the YGR021protein does not seem essential for respiration in yeast.[29]

Defects of Elongation Factors. Another important player during mitochondrial protein biosynthesis is the group of elongation factors. The mitochondrial EF-Tu forms a ternary complex with tRNA and GTP and promotes the binding of tRNA to the ribosome. Following codon:anticodon interactions, an EF-Tu:GDP complex is released from the ribosome. Elongation factor Ts binds to the EF-Tu:GDP complex and promotes the exchange of GDP for GTP, regenerating the active EF-Tu:GTP complex. Both mitochondrial EF-Tu and elongation factor Ts are encoded in the nuclear genome, synthesized in the cell cytoplasm, and subsequently imported into the mitochondrion.[40]

A few patients have been described as having mutations in genes encoding components of the mitochondrial translation elongation machinery, including elongation factor EF-Tu (*TUFM*), EF-Ts (*TSFM*), and EFG1 (*GFM1*), as shown in Figure 1B. These patients have severe disease, leading to early fatality, and show combined mitochondrial complex deficiencies. The single patient with mutations in *TUFM* showed lactic acidosis, a diffuse cystic leukoencephalopathy, polymicrogyria, and mild liver involvement. The clinical course of 1 child with mutations in the *GFM1* gene was dominated by neonatal lactic acidosis and rapidly evolving neurological impairment resembling Leigh syndrome, without any symptoms of hepatic failure. Another patient with *GFM1* mutations suffered postnatal hepatoencephalopathy.[40] A similar, ultimately lethal mutation in *TSFM* (namely, R312W) has been associated with encephalomyopathy in 1 neonate and hypertrophic cardiomyopathy in an unrelated infant. This mutation is predicted to disrupt important interactions between mitochondrial EF-Tu and EF-Ts, and the phenotypic differences might result from mutation-specific effects on the stability of the protein in different tissues. Differences in the abundance of other elongation factors have been suggested to have a role in this variability.[40,41]

Defects of MRPS16 and MRPS22. Of all 81 human mitochondrial ribosomal proteins, mutations have been found in only 2, *MRPS16* and *MRPS22* (Figure 1B).[42,43] Both defects resulted in a marked decrease in the 12S rRNA transcript level, probably caused by impaired assembly of the mitoribosomal small subunit, generating unincorporated and unstable 12S rRNA. Indeed, lack of MRP results in the failure to assemble parts of small subunits of the mitoribosome, and subsequent degradation of its components.[44] Clinical manifestations include agenesis of the corpus callosum, dysmorphism, hypertrophic cardiomyopathy, and fatal neonatal lactic acidosis. It is self-intuitive that the molecular defect is extremely severe for the developing brain.

Defects of Amino-acyl-tRNA Synthetases (RARS2, DARS2, and YARS2). To guarantee fidelity in translation, it is important to attach the right amino acid to the tRNA and to ensure that the tRNA recognizes, through its anticodon, the correct codon in the ribosomal A-site. Incorporation of an incorrect amino acid into the nascent polypeptide could cause misfolding and production of defective or dominant interfering proteins. Amino acids are attached to tRNA by amino-acyl-tRNA synthetases, each of which is specific for a single amino acid. However, as there can be several codons and several different tRNA for a single amino acid, an amino-acyl-tRNA synthetase can "charge" several different tRNA (Figure 2). If this function were defective, certain codons would become ambiguous, resulting in the synthesis of misfolded proteins, which could aggregate to form inclusions and induce further protein misfolding. Misfolding and protein aggregation are thought to underlie several neurodegenerative disorders.[40] This is the case for both cytoplasmic and mitochondrial protein synsthesis. In particular, mutations in 3 genes encoding cytoplasmic aminoacyl-tRNA synthetases (*GARS, YARS*, and *AARS*) have been implicated in Charcot-Marie-Tooth (CMT) disease, primarily associated with an axonal pathology (CMT2D, OMIM 601472; CMT2N, OMIM 613287) or intermediate forms (CMTDIC, OMIM 608323). One patient has been described as harboring recessive mutations in the *KARS* gene in association with intermediate CMT-like phenotype (CMTRIB type, OMIM 613641).[45]

Mutations in the mitochondrial arginyl (*RARS2)* and aspartyl-tRNA synthetases (*DARS2*; Figure 1B) are associated with severe encephalopathy with pontocerebellar hypoplasia and leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation, respectively.[40] The peculiar neuroimaging findings in both conditions drive a more correct molecular diagnosis, though clinical presentation can be heterogeneous, with most patients showing onset between 2-15 years of age. Most affected children present unsteady or delayed motor development, seizures, progressive onset of tremor, ataxia, dysarthria, and spasticity. An axonal peripheral neuropathy with distal weakness and decreased proprioception can also occur. Very recently, mutations in the gene encoding the mitochondrial tyrosyl-tRNA synthetase (*YARS2*; Figure 1B) have been associated with a clinical condition characterized by myopathy, lactic acidosis, and sideroblastic anemia. Given the grossly similar phenotype, it is possible that mutations in *PUS1* and *YARS2* share common pathogenetic events.[46] The reasons for the specific clinical phenotypes associated with mutations in the 3 aforementioned mitochondrial amino-acyl tRNA synthethases are presently unknown.

In the original report on RARS2 mutations, the $tRNA^{Arg}$ transcript was shown to be scarcely present, but almost fully acylated, in patients' fibroblasts. Presumably, small wild-type molecules can aminoacylate a small portion of $tRNA^{Arg}$, but tRNA could be unstable and subsequently impair mitochondrial protein synthesis. On the other hand, mutant DARS2 does not seem to affect mitochondrial translation and OXPHOS metabolism, though a clear reduction in aminoacylation activity is present. The reason is presently unclear.[44]

Conclusion

The diagnostic process in infantile disorders of oxidative metabolism is not too different from that employed for other diseases and includes patient and family history, physical and neurologic examination, routine and special laboratory tests, muscle biopsy for morphology and biochemistry, and molecular genetics screening.[47] A mitochondrial disease manifesting at or soon after birth is more likely to be associated with nDNA than with mtDNA mutations, but until very recently, our profound ignorance regarding the mechanisms underlying mitochondrial gene transcription and translation and the complex interaction between the "2 genomes" has limited our diagnostic power.

Mitochondrial DNA depletion syndromes and disturbances in the mitochondrial translation machinery have become an increasingly important cause of a wide spectrum of infantile and childhood-onset multisystem disorders. Depletion syndromes could result from any imbalance of the mitochondrial nucleotide pool available for mtDNA replication, as well as abnormalities in either the mitochondrial helicase or DNA polymerase.[18] Consistent with the different phenotypes, mtDNA depletion may affect specific tissue (most commonly, brain and muscle or liver) or multiple organs, including the heart and the kidney. Predictably, affected tissues show paucity of mtDNA-encoded translation products and multiple respiratory chain defects. More than 75% of these patients had onset during the first year of life, and the disease was rapidly fatal in most cases.[48,49] Moreover, though the components of the complicated mitochondrial protein-synthesis machinery are exclusively nuclear encoded, the majority of mutation affects correct translation of mtDNA-encoded subunits of the OXPHOS system and accounts for a still undetermined number of genetic defects. Indeed, there is still limited information on the many mitoribosomal proteins; the several tRNA maturation enzymes; the aminoacyl-tRNA synthetases; the translation initiation, elongation, and termination factors; and the predictably larger number of unidentified factors needed for ribosome assembly.[29,44] Certainly, further players in this scenario are soon likely to appear.[13] Far from being simply anecdotal, the recent progress in the molecular definition of a growing array of infantile mitochondrial disorders—all with the common molecular theme of mitochondria "lost in translation"—has widened our possibilities to reach a confirmatory molecular diagnosis and offered new understanding of mitochondrial biology. As an example, the tissue-specific regulation of these genes might give some explanation of how tissue specificity occurs.

More than 20 years after the discovery of pathogenic mutations in mtDNA, and 10 years into the "nDNA age" of mitochondrial medicine, we have still little understanding of how singular molecular defects cause different syndromes.[5] If conventional wisdom dictates that a common molecular theme in the process of mitochondrial replication or protein biosynthesis should be suspected—and we are unraveling the different pieces of a large mosaic—the clinical outcome should be a flood of ill-defined and overlapping syndromes, whereas most mutations result in well-defined and rather stereotypical presentations in clinical terms. This is no surprise, however, when one considers that the simple and "born-to be-free" mtDNA is subordinated to a chief-commander (the nDNA) that is exerting its power in an unexpectedly complex way. It is anticipated that an increase in high-throughput genomic sequencing approaches will reveal a higher and unsuspected degree of indirect genetic control of the nuclear over the mitochondrial genome.

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Author Contributions

Dr. Nogueira participated in the conception, organization, and execution of the research; review and critique of the statistical analysis; and writing, review, and critique of the manuscript. Dr. Carrozzo participated in the review and critique of the statistical analysis and the manuscript. Dr. Vilarinho participated in the design, execution, review, and critique of the statistical analysis; and in the review and critique of the manuscript. Dr. Santorelli participated in the conception and organization of the research; and in the review and critique of the statistical analysis and the manuscript.

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Chapter 13

Nuclear-Mitochondrial Intergenomic Communication Disorders

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Additional information is available at the end of the chapter

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1. Introduction

Mitochondria are dynamic subcellular organelles present in virtually all eukaryotic cells with numerous functions. The most important of these functions is production of ATP; however they play an important role in various metabolic and developmental processes such as calcium homeostasis, apoptosis and programmed cell death, just to mention some. Mitochondria produce ATP by means of the mitochondrial respiratory chain (MRC) and oxidative phosphorylation (OXPHOS) system, a series of five enzyme complexes embedded in the inner mitochondrial membrane. Mitochondrial disorders most often refer to the dysfunction of OXPHOS system leading to deficiency in the ATP production. They are a group of genetically and phenotypically heterogeneous disorders with an incidence estimated to be between 1:5,000 and 1:10,000 live births [1].

MRC is the result of the interplay of two physically and functionally separated genomes, the nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Thirteen of the key structural polypeptides that constitute the multimeric subunits of the respiratory chain complexes are mtDNA encoded, in addition two ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) that are required for initiating translation and protein synthesis [2]. Approximately 90 of the remaining proteins that make up the respiratory chain complexes are encoded by nDNA. Therefore, although human mtDNA encodes the basic machinery for protein synthesis, it depends entirely on the nucleus for the provision of enzymes for replication, repair, transcription, and translation. This dependency lies at the heart of several newly recognized human diseases that are characterized by secondary abnormalities of mtDNA.

The crosstalk between the two genomes is crucial for the cellular regulation of mtDNA integrity and copy number and correct mitochondrial protein production therefore mutations in genes involved in mitochondrial replication and maintenance can disrupt the integrity of the mitochondrial genome, causing inter-genomic communication disorders. Multiple deletions, depletion of mtDNA or a combination of both phenomena (qualitative/quantitative lesions) in critical tissues, are the hallmarks of these disorders.

The focus of this chapter is to review the clinical and molecular etiologies of nuclear defects involved in mtDNA stability and in mitochondrial protein synthesis. The overview done here will hopefully provide insights towards best diagnostic strategies of mitochondrial cross–talk disorders, being useful for clinicians when facing similar cases. Additionally we will present a diagnostic algorithm for these diseases based on our knowledge.

2. Clinical manifestations of disorders affecting mtDNA integrity

Maintenance of mtDNA is controlled by an intricate homeostatic network, whose effectors are the various components of the mitochondrial replicosome and the many enzymes and carrier proteins that provide the mitochondrion with a balance supply of deoxyribonucleotides (Figure 1). As all of the factors are nDNA encoded, it is not surprising that mutations in genes involved in mitochondrial replication and maintenance can disrupt the integrity of the ''tiny'' mitochondrial genome [3] leading to multiple deletions or depletion [4]. The mitonuclear crosstalk has gained increased relevance in the past years and since then many genes have been identified as being involved in these diseases.

In the following section we will briefly review the clinical manifestations of both these group of disorders.

2.1. mtDNA multiple deletion syndromes

Mitochondrial diseases associated with the presence of multiple deletions of mtDNA are mostly autosomal dominant, occurring most often in adulthood. The size and terminals deletions are variable from one individual to another within the same family.

The main clinical manifestations associated with multiple deletions are:

i. **PEO** (autosomal dominant or recessive **P**rogressive **E**xternal **O**phthalmoplegia). The most common clinical features include adult-onset of weakness of the external eye muscles, bilateral ptosis, proximal muscle weakness wasting and exercise intolerance. Additional symptoms are variable, and may include cataracts, hearing loss, sensory axonal neuropathy, ataxia, depression, hypogonadism, and Parkinsonism. Less common features include mitral valve prolapse, cardiomyopathy, and gastrointestinal dysmotility. Both autosomal dominant and autosomal recessive inheritance can occur; autosomal recessive inheritance is usually more severe [5,6]. The multiple deletions associated with PEO are exclusively found in muscle tissues of patients.
ii. **SANDO** (**S**ensory **A**taxic **N**europathy, **D**ysarthria and **O**phthalmoparesis) is an autosomal recessive systemic disorder characterized mainly by adult onset of sensory ataxic neuropathy, dysarthria, and ophthalmoparesis. The phenotype varies widely, even within the same family, and can include myopathy, seizures, and hearing loss, but the common clinical feature appears to be sensory ataxia [7].

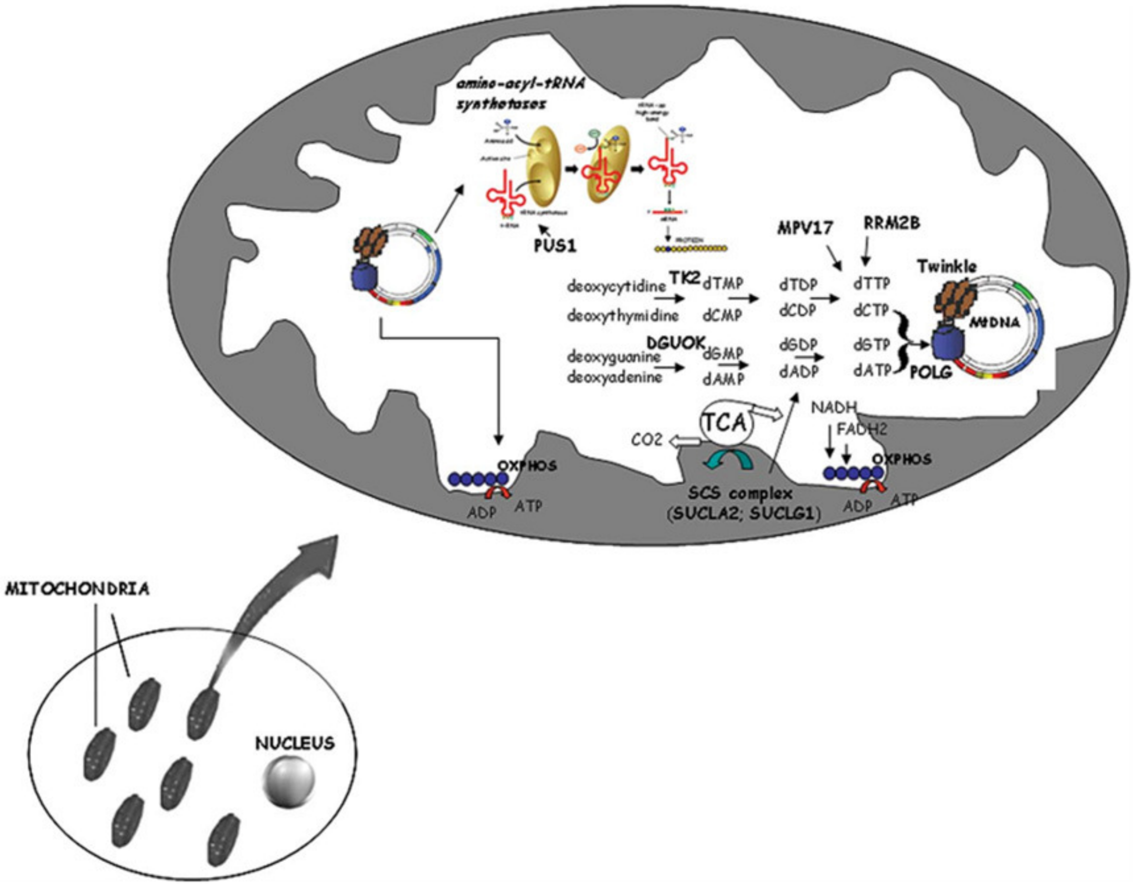

Figure 1. Schematic overview of the mitochondrion and the mitochondrial disease genes involved in intergenomic communication disorders. Zooming in on the mitochondrion allows identification of genes (namely, *POLG* and *C10orf2- Twinkle*) thought to be involved in replication of mitochondrial DNA (mtDNA); those assumed to affect the metabolism of the mitochondrial deoxynucleotide (dNTP) pool (via progressive phosphorylations of deoxythymidine, deoxycytidine, deoxytadenine, and deoxiguanosine); and those belonging to the tricarboxylic acid cycle and affecting the respiratory chain complexes (OXPHOS). Moreover, the supposed role of genes involved in the complex machinery of mitochondrial protein synthesis (including the aminoacyl-tRNA synthetases) is illustrated. This figure was kindly provided by Prof. Filippo M. Santorelli.

iii. **MNGIE** (**M**itochondrial **N**euro**G**astro**I**ntestinal **E**ncephalomyopathy), an autosomal recessive disorder clinically characterized by onset between the second and fifth decades of life, PEO, gastrointestinal dysmotility (often pseudo-obstruction), cachexia, diffuse leukoencephalopathy, peripheral neuropathy and early death. Mitochondrial DNA abnormalities can include depletion, multiple deletions, and point mutations [8].
iv. **SCAE** (**S**pino**C**erebellar **A**taxia – **E**pilepsy syndrome) disorder similar to SANDO but with a higher frequency of migraine headaches and seizures [9].

2.2. mtDNA depletion syndromes

Quantitative alterations are characterized by depletion of mtDNA. Mitochondrial DNA depletion syndrome (MDS) comprises a heterogeneous group of autosomal recessive disorders, all having the same molecular end result, low mtDNA amount in specific tissues. MDS are a group of rare and devastating diseases that manifest typically, although not exclusively, soon after birth, determining early death usually in infancy or early childhood. MDS differs from other respiratory chain disorders, as most often it may manifest solely in a specific organ (most commonly muscle or liver) [10]. However, it may occur that multiple organs, including heart, brain, and kidney are affected [11]. An extensive review on MDS was recently published [12].

Three major clinical categories can be recognized however, the clinical phenotypes are heterogeneous, overlapping and ever expanding [10,13]:

i. **Hepatocerebral MDS** is most probably the most common variant of MDS; Onset of symptoms is between birth and 6 months; death usually occurs within one year of age. The most common symptoms and signs include persistent vomiting, failure to thrive, hypotonia and hypoglycemia associated with progressive neurological symptoms. Histological changes on liver biopsy include fatty degeneration, bile duct proliferation, fibrosis, and collapse of lobular architecture. Reduced COX histochemistry and combined deficiency of mtDNA encoded MRC complexes were found in the liver of a few patients.

   A peculiar form of hepatocerebral MDS is Alpers-Huttenlocher syndrome, an early onset, fatal disease, characterized by hepatic failure, intractable seizures, evolving into *epilepsia partialis continua*, and global neurological deterioration. The liver dysfunction is usually progressive as well, evolving from microvesicular staetosis with bile duct proliferation into cirrhosis and chronic liver failure.

ii. **Myopathic MDS** typically onset of symptoms usually occur in the first year of life with feeding difficulty, failure to thrive, hypotonia, muscle weakness and occasionally PEO. Death is usually due to pulmonary insufficiency and infections, but some patients survive into their teens [14,15]. Muscle biopsy may show proliferation of mitochondria, which can increase with age, and patchy or diffuse COX deficiency. Biochemical defects of all mtDNA-related respiratory chain complexes are always present in muscle mitochondria. Serum CK levels may be variably elevated [4].

iii. **Encephalomyopathic MDS** is characterized by infantile onset of hypotonia with severe psychomotor retardation, high lactate in blood, progressive neurologic deterioration, a hyperkinetic-dystonic movement disorder, external ophthalmoplegia, deafness, generalized seizures and variable renal tubular dysfunction. Brain MRI was suggestive of Leigh syndrome [11].

3. Molecular etiologies of disorders affecting mtDNA integrity

In the next sections we will mention the genes identified so far, to be responsible with these disorders. Table 1 summarizes the mutations described and the associated phenotypes.

Gene	Phenotype	Mutation Type							
		M/N	Sp	Sd	Si	Sid	Gd	Gi	Gr
POLG	PEO	61		1	2				
	Alpers	37	5	2	6	2	3		
	MDS	6		1					
	Encephalopathy	3							
	Mitochondrial spinocerebellar ataxia and epilepsy	1							
	SANDO	1			1				
	POLG deficiency	29	1	2	2				
	OXPHOS deficiency	2							
	Ataxia	6	1						
	Epilepsy	2		1					
	Complex I deficiency	1							
	Others	18	1						
POLG2	PEO	1							
	Mitochondrial disease	7		1				1	
SLC25A4	PEO	5							
	Mitochondrial myopathy & hypertrophic cardiomyopathy	1	1	1	1				
SLC25A3	Muscular hypotonia & hypertrophic cardiomyopathy	1	1						
C10orf2	PEO	34				1		1	
	MDS	1							
	Spirocerebellar ataxia, infantile onset	2							
	Cholestatic liver disease	1							
	Encephalopathy	1							
	Ocular myopathy	1							
	Complex I deficiency	1							
	Dementia	1							
	Reduced expression	1							
Tymp	MNGIE	47	11	11	6	1			
TK2	MDS	20	1	2	9	1	1		1
	Epileptic encephalomyopathy				1				
	MDS with hearing loss				1				
	PEO	1							
DGUOK	MDS	28	6	9	4		1		
RRM2B	MDS	12	2	1					
	PEO	7		1	1				
	MNGIE	2							
	KSS	1							

Gene	Phenotype	Mutation Type							
		M/N	Sp	Sd	Si	Sid	Gd	Gi	Gr
MPV17	Altered p53 binding, association with		1						
	MDS – hepatocerebral	7	1	1		1	2		
	Liver failure in infancy			2					
	Lethal hepatopathy & leukodystrophy		1						
SUCLA2	MDS – encephalomyopatic & methylmalonic ac.	4	1			1			1
SUCLG1	Succinyl-coenzyme A synthetase deficiency	4	1	1					
	Lactic acidosis with mitochondrial DNA depletion	2		1					
	Neonatal lactic acidosis with methylmalonic aciduria	2							
	Lactic acidosis, fatal infantile		1						
	Mitochondrial hepatoencephalomyopathy	1							
PUS1	Mitochondrial myopathy and sideroblastic anaemia	2							
	Sideroblastic anaemia	1							
TRMU	Infantile liver failure, increased risk	6	1	1					
	Combined OXPHOS deficiency				2				
	Phenotype modifier	1							
	Respiratory chain deficiency, reversible infantile		1						
LRPPRC	Cytochrome c oxidase deficiency	1				1			
TACO1	Cytochrome c oxidase deficiency				1				
TUFM	Combined OXPHOS deficiency	1							
TSFM	Combined OXPHOS deficiency	2							
GFM1	Combined OXPHOS deficiency	8		2					
MRPS16	MRC disorder	1							
RARS2	Pontocerebellar hypoplasia	3	1	1					
DARS2	Leukoencephalopathy, brain & spine involvement, lactate elevation	15	11	1		2	1		
	Episodic ataxia, exercise-induced	1							
YARS2	MLASA syndrome	1							

Table 1. Mutations types described in genes involved in mtDNA integrity and mitochondrial translation and associated clinical phenotype (M/N- missense/nonsense; Sp- splicing; Sd- small deletions; Si- small insertions; Sid- small inddels; Gd- gross deletions; Gi- gross insertions; Gr- gross rearrangements) - source HGMD Professional database www.hgmd.cf.ac.uk/.

3.1. Genes involved in mitochondrial replisome

3.1.1. POLG

Human mitochondria contain a single DNA polymerase, Polymerase gamma (POLγ), nuclear encoded and solely responsible for mtDNA replication and repair in mitochondria. POLγ is composed of a catalytic subunit, POLγA, which possesses both polymerase and proofreading exonuclease activities and an accessory subunit, POLγB, which increases enzyme processivity [16]. The POLγ holoenzyme functions in conjunction with the mitochondrial DNA helicase and the mitochondrial single-stranded DNA- binding protein to form the minimal replication apparatus [17]. It was generally accepted that mutations within the mtDNA were the major cause of mitochondrial diseases; however this view is changing as several of these have been linked to ineffective mtDNA replication by POLγ.

Mutations affecting the catalytic subunit POLγA, encoded by the nuclear gene *POLG* are a major cause of mitochondrial disease, being highly heterogeneous – PEO, Parkinsonism, AHS, MNGIE, SANDO and SCAE- and usually is associated with multiple mtDNA deletions [18]. POLG mutations have been shown to be associated with all types of inheritance. The unique features of mitochondrial physiology are in part responsible for this variability but *POLG* structure and function add to the riddle of how one gene product can demonstrate autosomal recessive and autosomal dominant transmission. POLγA is a key player in mtDNA maintenance that is absolutely necessary for mtDNA replication from an early stage in embryogenesis [19]

In adPEO due to *POLG* mutations (most frequent), prominent features are severe dysphagia and dysphonia, and, occasionally, a movement disorder including Parkinsonism, cerebellar dysfunction, and chorea. Recessive mutations of *POLG* are responsible for sporadic and arPEO, as well as the syndromes referred above. Mutations in this gene can be also associated to the hepatocerebral form of MDS, namely AHS [18].

The *POLG* gene is located at chromosome 15, comprises 23 exons spanning 18.55 Kb. The gene was identified in 1996 [20] but only in 2001 the first pathogenic mutation was described. Since then more than 150 mutations have been reported and POLG gene is considered a hot-spot for mutations in mitochondrial diseases [21].

3.1.2. POLG2

MtDNA is replicated by DNA polymerase gamma, which is composed of a 140-kD catalytic subunit (encoded by *POLG*) and a 55-kD accessory subunit (*POLG2*). The accessory subunit increases enzyme processivity therefore it is not surprising that failure in this processivity leads to the accumulation of mtDNA deletions.

The *POLG2* gene is located at chromosome 17, comprises 8 exons spanning 19.28 Kb. In 2006 the first pathogenic mutation was described as being a cause of adPEO [22]. Since then, 10 mutations in *POLG2* have been reported.

3.1.3. C10orf2 (Twinkle)

The mitochondrial helicase/primase encoded by *C10orf2* gene is also responsible for the adPEO [23]. Mutations in *C10orf2* may be of variable severity, being associated with clinical presentations ranging from late-onset ''pure'' PEO, to PEO complicated by proximal limb and facial muscle weakness, dysphagia and dysphonia, mild ataxia, and peripheral neuropathy. Recessive *C10orf2* mutations were also described in patients with hepatocerebral form of MDS [24].

The *C10orf2* gene is located at chromosome 10; it comprises 5 exons spanning 6.38 kb. The first pathogenic mutation was reported in 2001 [23] to be associated with PEO and since then 45 pathogenic mutations have been reported.

3.2. Genes involved in the synthesis and supply of nucleotide pools

3.2.1. SLC25A4

This gene, coding for the muscle-heart-specific mitochondrial adenine nucleotide translocator (ANT) is a member of the mitochondrial carrier subfamily of solute carrier protein genes [25]. ANT is the most abundant mitochondrial protein and in its functional state, it is a homodimer of 30-kD subunits embedded asymmetrically in the inner mitochondrial membrane. The dimer forms a gated pore through which ADP is moved from the matrix into the cytoplasm. There are three recognized isoforms of this protein.

Mutations in this gene have been shown to be responsible for the adPEO and have been also associated with a relatively mild, slow progressive myopathy, with little or no extramuscular symptoms.

The *SLC25A4* gene was identified in 2000 [25], it is located at chromosome 4, comprises 4 exons spanning 4.04 Kb. The first pathogenic mutations were described in 2000 and since then only seven mutations have been reported (most of them associated with PEO).

3.2.2. SLC25A3

The *SLC25A3* gene codes for a mitochondrial phosphate carrier. A defect in this mitochondrial phosphate carrier has been described in two children with hypertrophic cardiomyopathy, muscular hypotonia, severe growth retardation and death in the first year of life [26].

The gene is located at chromosome 12, comprises 7 exons spanning 8.37 Kb. The first pathogenic mutations were described in 2007 [26] and since then only one more mutation has been reported.

3.2.3. Tymp (ECGF1)

The *Tymp* gene, responsible for MNGIE (Mitochondrial NeuroGastroIntestinal Encephalomyopathy), encodes the enzyme thymidine phosphorylase (TP), which is involved in pyrimidines catabolism. Defects of TP result in systemic accumulation of thymidine and deoxyuridine, which leads to deoxynucleotide pool imbalance and mtDNA instability, resulting in the presence of multiple deletions and partial depletion of muscle mtDNA [27].

The *Tymp* gene is located at chromosome 22 it comprises 10 exons spanning 4.3 kb. The first pathogenic mutations were described in 1999 [27] and since then 65 mutations have been described as being associated with MNGIE.

3.2.4. TK2

Thymidine kinase (TK2) is an intramitochondrial pyrimidine nucleoside kinase that phosphorylates deoxynucleotides (dNTPs), such as: deoxythymidine, deoxycytidine, and deoxyuridine, thereby participating in the salvage pathway of deoxynucleotide synthesis in the mitochondria [28]. Mitochondrial dNTPs pools arise either through active transport of cytosolic dNTP or through salvage pathways. Both pathways are essential for the replication of mtDNA, since the mitochondrion is unable to synthesize dNTPs *de novo*. Mutations in the *TK2* gene on chromosome 16q22 affect primarily muscle tissue, with little or no effect on the liver, brain, heart, or skin. The typical manifestation of *TK2* mutations is a severe, rapidly progressing myopathy of infantile or childhood onset. The disease course is rapidly progressive, leading to respiratory failure and death in months or years, but milder phenotypes with slower progression and longer survival have been reported [10]. Since the first mutation was described in 2001 [29], approximately 25 different pathogenic mutations in *TK2* have been published so far, either as recessive homozygous or compound heterozygous mutations, and phenotypes may be explained by variable degrees of residual activity of the mutant enzymes.

3.2.5. DGUOK

Deoxyguanosine kinase is a 2-deoxyribonucleoside enzyme that catalyzes the first step of the mitochondrial deoxypurine salvage pathway, the phosphorylation of purine deoxyribonucleosides into the corresponding nucleotides deoxyguanosine and deoxyadenosine necessary for the maintenance of mitochondrial dNTPs pools [11,30]. The typical phenotype of mutations in the *DGUOK* gene, on chromosome 2p13, is characterized by neonatal onset of progressive liver disease and feeding difficulties, usually with neurological dysfunction (hypotonia, nystagmus, and psychomotor retardation), by the age of 3 months. Peripheral neuropathy and renal tubulopathy have occasionally been reported [31]. Depletion of mtDNA has been documented only in the liver and results in combined respiratory chain deficiencies in the liver, whereas the amount of mtDNA is usually normal in muscle and fibroblasts. Histological analyses of the liver biopsy show variable findings, typically microvacuolar steatosis, cholestasis, fibrosis, and cirrhosis. In most cases, there is a rapidly progressive liver disease and neurological deterioration, with death occurring by the age of 12 months or shortly thereafter [32]. The first pathogenic mutations was reported in

2001 [33], since then more than 80 affected patients from approximately 50 families have been reported, and over 40 different *DGUOK* mutations have been identified [10]. The infantile hepatocerebral form of MDS is the almost invariable clinical presentation. Genotype-phenotype correlation studies show that patients who harbor null mutations usually have early onset liver failure and significant neurological disease, including hypotonia, nystagmus, and psychomotor retardation, and death before two years of age. Patients carrying missense mutations usually have isolated liver disease, a better prognosis, and longer survival.

3.2.6. RRM2B

The *RRM2B* gene on chromosome 8q23 encodes the small subunit of p53-inducible ribonucleotide reductase, a heterotetrameric enzyme responsible for *de novo* conversion of ribonucleoside diphosphates into the corresponding deoxyribonucleoside diphosphates that are crucial for DNA synthesis [34]. The enzyme is the main regulator of the nucleotide pools in the cytoplasm, and its small subunit is expressed in postmitotic cells, where it probably has a key function in maintaining the mitochondrial dNTPs pools for mtDNA synthesis. Mutations in *RRM2B* usually result in hypotonia, lactic acidosis, failure to thrive, and tubulopathy in the first months of life. The disease has a rapid progression and leads to death in a few months. The associated complex phenotype suggests that the consequences of a defective mitochondrial dNTPs pools can vary dramatically depending on the residual amount of the functional enzyme. Recently, it has been shown that inactivating mutations in *RRM2B* also cause severe neonatal or infantile forms of mtDNA depletion, with profound reduction of mtDNA copy numbers in skeletal muscle [34]. The first pathogenic mutation was reported in 2007 [34] and since then 26 mutations have been described.

3.2.7. MPV17

The *MPV17* gene is located on chromosome 2p23-p21 and encodes a mitochondrial inner membrane protein of unknown function recently recognized as responsible for mtDNA depletion. The clinical presentation is that of severe liver failure, hypoglycemia, growth retardation, neurological symptoms, and multiple brain lesions during the first year of life [35]. Marked mtDNA depletion in the liver is the molecular hallmark associated with multiple defects of respiratory chain complexes. Normal or mildly reduced levels of both mtDNA content and respiratory chain enzyme activities were also found in muscle [36]. Histological analyses of the liver have revealed swollen granular hepatocytes, microvesicular steatosis, and focal pericellular and periportal fibrosis. Since the first mutation was described in 2006 [37], about 15 different mutations have been reported in infantile-onset hepatocerebral syndrome and in Navajo neurohepatopathy, which is an autosomal recessive multisystem disorder found in the Navajo of the southwestern United States [30]. Three main subtypes are to be considered: infantile-onset (before 6 months) and childhood-onset (before 5 years) forms with hypoglycemic episodes and severe progressive liver dysfunction requiring liver transplant, and a 'classic'' form with moderate hepatopathy and progressive sensorimotor axonal neuropathy. The three forms are also associated with variable degrees of demyelination in both the central and the peripheral nervous system.

3.2.8. SUCLA2 and SUCLG1

Succinyl CoA synthase is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinate and ATP or GTP from succinyl-CoA and ADP in the tricarboxylic acid cycle. This enzyme is made up of two subunits, a and b, encoded by *SUCLG1* on chromosome 2p11 and *SUCLA2* on 13q12, respectively. Mutations in *SUCLA2* and *SUCLG1* cause an encephalomyopathic form of infantile mtDNA depletion syndrome, but *SUCLG1* can also cause a very severe disorder with antenatal dysmorphisms, neonatal metabolic crisis, and early death, probably depending on the lower residual amount of the protein [38,39]. A useful diagnostic clue in Succinyl CoA synthase disorders of succinyl CoA synthase is a ''mildly'' elevated urinary methylmalonic acid, which is detected in all patients, and presence of tricarboxylic acid cycle intermediates (methylcitrate, lactate, carnitine esters, 3-hydroxyisovalericacid) in most cases. Some patients die as infants (sudden infant death syndrome), but some of them have a longer survival. The clinical features of patients with mutations in these genes include early childhood hypotonia, developmental delay, and almost invariably, progressive dystonia and sensorineural deafness. *SUCLA2* and *SUCLG1* mutations seem to disrupt an association between succinyl CoA synthase and mitochondrial nucleoside diphosphate kinase, resulting in an unbalanced mitochondrial dNTP pool and eventually, mtDNA depletion in muscle. The first pathogenic mutations were reported in 2005 [40] and 2007 [41] in *SUCLA2* and *SUCLG1*, respectively and since then few mutations have been described.

3.3. Genes involved in mitochondrial translation

Mendelian diseases characterized by defective mitochondrial protein synthesis and combined respiratory chain defects have also been described in infants and are associated with mutations in nuclear genes that encode components of the translational machinery, such as those encoding elongation factors, aminoacyl-tRNA synthetases, or even mtDNA encoded tRNA [12]. Mitochondria contain a separate protein-synthesis machinery to produce the polypeptides encoded in mtDNA, and many mtDNA disease mutations affect this machinery. This group of disorders is highly heterogeneous and usually shares a combined disorder of respiratory chain complexes.

3.3.1. Genes involved in mitochondrial translation factors

3.1.1.1. PUS1

The Pseudouridine synthase 1 (*PUS1*) gene on chromosome 12q24 encodes an enzyme that converts uridine into pseudouridine at several cytoplasmic and mitochondrial tRNA positions and thereby improves translation efficiency in the cytosol as well as the mitochondrion. Thus, *PUS1* is not part of the translation machinery, but it is required for protein synthesis because of its function in posttranscriptional modification of tRNA. Mutations in *PUS1* are responsible for the rare myopathy, lactic acidosis, sideroblastic anemia syndrome and sometimes include mental retardation. The first pathogenic mutation was reported in 2004 [42] and since then few mutations have been described.

3.1.1.2. TRMU

The *TRMU* gene on chromosome 22q13 encodes an evolutionarily conserved protein involved in mitochondrial tRNA modification and is important for mitochondrial translation. Defects in tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (TRMU), a mitochondria specific enzyme that is required for the 2-thiolation on the wobble position of the tRNA anticodon, result in reduced steady-state levels of 3 tRNA (tRNALys, tRNAGln, and tRNAGlu) and consequently, impaired mitochondrial protein synthesis [43,44]. Recently, mutations in *TRMU* were detected in patients with acute liver failure in infancy [44].

3.1.1.3. LRPPRC

The *LRPPRC* gene is located on chromosome 2p21. Leucine-rich PPR-motif containing protein has been suggested to function together with heterogeneous nuclear ribonucleoprotein K and RNA polymerase in coupling the mitochondrial transcription and translation machineries [45]. Mutations in *LRPPRC* lead to the French-Canadian subtype of Leigh syndrome, associated with a profound deficiency of complex IV of the OXPHOS system [46]. Patients exhibit neonatal or infantile onset hypotonia and psychomotor delay, and bilateral hyperluciencies of basal ganglia, like other more common forms of Leigh syndrome. The first pathogenic mutation was reported in 2003 [46] and since then one more mutation has been described.

3.1.1.4. TACO1

TACO1 represents the first specific mammalian mitochondrial translational activator, opening the possibility to a new class of proteins controlling efficiency of mitochondrial translation. Mutations in *TACO1*, located on chromosome 17q.6, are responsible for a relatively late-onset Leigh syndrome (onset range 4-13 years) characterized by short stature, mental retardation with autistic-like features, and a slowly progressive array of motor symptoms related mainly to basal ganglia involvement [47,48]. Only one mutation was described to date [47].

3.1.1.5. TUFM, TSFM and GFM1

Another important player during mitochondrial protein biosynthesis is the group of elongation factors. The mitochondrial EF-Tu forms a ternary complex with tRNA and GTP and promotes the binding of tRNA to the ribosome. A few patients have been described as having mutations in genes encoding components of the mitochondrial translation elongation machinery, including elongation factor EF-Tu (TUFM), EF-Ts (TSFM) and EFG1 (GFM1). These patients have severe disease, presenting neonatal lactic acidosis and neurological impairment resembling Leigh syndrome, leading to early fatality. The first pathogenic mutations in these genes were reported recently [49,50,51] and since then few mutations have been described.

3.1.1.6. MRPS16 and MRPS22

Of all 81 human mitochondrial ribosomal proteins (MRPs), mutations have been found in only two, MRPS16 and MRPS22 [52,53]. Both defects resulted in a marked decrease in the 12S rRNA transcript level, probably caused by impaired assembly of the mitoribosomal small subunit, generating unincorporated and unstable 12S rRNA. Indeed, lack of MRPs results in the failure to assemble parts of small subunits of the mitoribosome, and subsequent degradation of its components [54]. Clinical manifestations include agenesis of the *corpus callosum*, dysmorphism, hypertrophic cardiomyopathy, and fatal neonatal lactic acidosis. The first pathogenic mutations were reported in 2004 [52] and in 2007 [53], and since then few mutations have been described.

3.3.2. Genes involved in mitochondrial aminoacyl tRNA synthetases

3.3.2.1. RARS2, DARS2, and YARS2

To guarantee fidelity in translation, it is important to attach the right amino acid to the tRNA and to ensure that the tRNA recognizes, through its anticodon, the correct codon in the ribosomal A-site. Incorporation of an incorrect amino acid into the nascent polypeptide could cause misfolding and production of defective or dominant interfering proteins. Amino acids are attached to tRNA by amino-acyl-tRNA synthetases, each of which is specific for a single amino acid. However, as there can be several codons and several different tRNA for a single amino acid, an amino-acyl-tRNA synthetase can ''charge'' several different tRNA. If this function is defective, certain codons will become ambiguous, resulting in the synthesis of misfolded proteins, which could aggregate to form inclusions and induce further protein misfolding. Mutations in the *RARS2* and *DARS2* were recently described [55,56,57] and are associated with severe encephalopathy with pontocerebellar hypoplasia and leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation, respectively, with most patients showing onset between 2-15 years of age [56]. Very recently, mutations in the gene encoding the mitochondrial *YARS2* have been associated with a clinical condition characterized by myopathy, lactic acidosis, and sideroblastic anemia [54].

4. Diagnostic approaches for intergenomic communication disorders

Suspicion of intergenomic communication disorders arising from clinical presentation may range from well defined syndromes to unspecific multisystemic phenotype, where neurological involvement is usually present.

Establishing a specific diagnosis in a patient with suspected mendelian disease is a challenging task that requires the integration of clinical assessments, family history, biochemical testing and histopathological examination. It is important to obtain the appropriate biochemical and/or clinical information before starting any molecular investigations so that molecular diagnosis can be successfully.

Biochemical determination of mitochondrial respiratory chain complexes is important for delineating the molecular approach in particular in patients without a specific neurological syndrome. As mtDNA encodes for subunits of respiratory chain complexes I, III, IV and the ATP-synthase, mtDNA depletion causes a combined respiratory chain deficiency of all complexes, except complex II. Biochemical analysis of the muscle respiratory chain enzyme activities may, however, be normal, if skeletal muscle is not among the affected tissues, e.g., in MDS of the brain or liver. Southern analysis or quantitative real-time polymerase chain reaction are two methods that simultaneously detects mtDNA deletion(s) and quantify total mtDNA content. In both approaches, mtDNA amount is compared to a specific nuclear reference gene. A prerequisite for correct interpretation of mtDNA amount is to consider the dynamic nature of mtDNA amount in different ages and tissues, and therefore to establish carefully age-matched control materials [58]. A reduction in mtDNA copy number to 60-65% of age-matched controls has been established for an empirical cut-off level for MDS diagnosis, but especially in children, the reduction may be severe (80-90%). Biochemical data, such as lactate, pyruvate, alanine, organic acid profiles as well as neuroimaging findings are also important clues for the diagnosis of these disorders. Some diagnostic clues exist for specific gene defects: serum creatine kinase (CK) is elevated in *TK2* defects, serum thymidine in *TYMP* defects and urine methylmalonic acid and methylcitrate in *SUCLA2* and *SUCLG1* defects [10].

The *POLG* gene seems to be the most frequently mutated nuclear gene in cases of mitochondrial disease therefore in cases of normal mtDNA testing and clinical signs such as nonspecific hypotonia, developmental delay, epilepsy and progressive liver disease *POLG* gene investigation should be considered. Valproate-induced liver toxicity in *POLG* and *C10orf2*-MDS emphasizes the importance of diagnosing these patients, who usually suffer from severe treatment-resistant epilepsy [59]. We suggest *POLG* analysis before valproate treatment for such children and adolescents, whose first epileptic attack develops to a status epilepticus of unknown cause.

Based on our practice, we present a testing algorithm for establishing an accurate diagnosis for these diseases (Figure 2).

5. Therapeutic considerations

The management of mitochondrial disease is largely supportive as no curative therapy is available. Palliative/supportive treatment with vitamins, cofactors and respiratory substrates have been used, but with poor efficacy. In the last years several approaches have been tried and the enhancement of mitochondrial biogenesis has emerged as an exciting therapeutic possibility. The enhancement of mitochondrial biogenesis might restore mitochondrial function in a variety of other contexts.

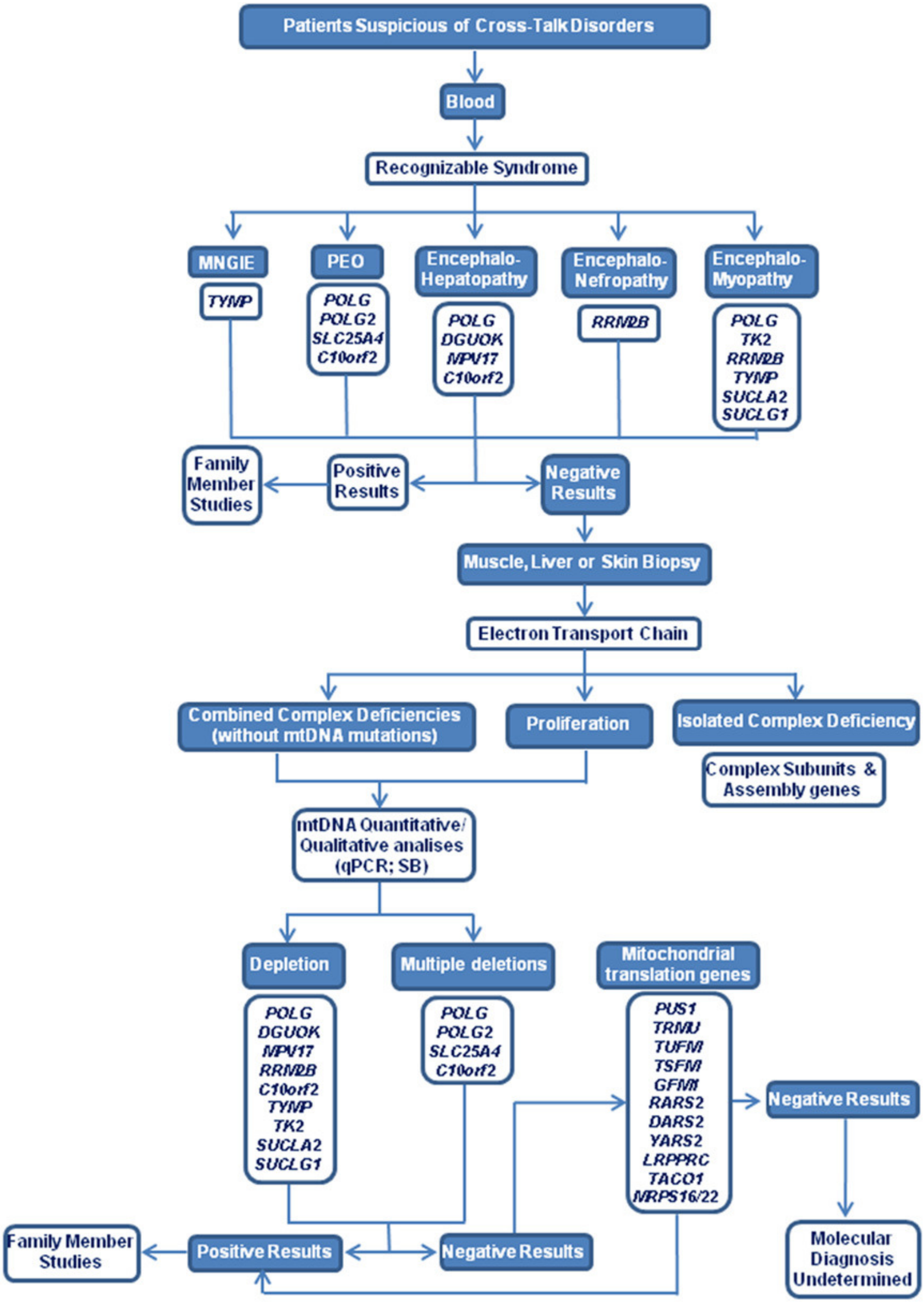

Figure 2. Diagnostic algorithm for intergenomic communication disorders, based on clinical and biochemical information.

What has been noticed is that for every case there is a different strategy. For example liver transplantation may be beneficial to patients with hepatopathy caused by *DGUOK* mutations if no neurological symptoms have developed. However, significant hypotonia, psychomotor retardation or nystagmus should be contraindications for the liver transplantation [60]. In patients with *MPV17*, liver transplantation has increased quality and years to life for some patients [61,62], but the patients have developed neurological symptoms. Some children with *POLG* mutations have received a liver transplant after valproate-induced liver failure, and although it has rescued their liver function, neurological outcome has been unfavorable [63,64].

In patients with *MPV17* mutations, a controlled diet avoiding hypoglycemias were suggested to slow down the progression of liver impairment and be useful in supportive care [65]. Some improvement of liver functions in a patient with *MPV17* mutations was gained by treating them with succinate or coenzyme-Q10 together with a lipid-rich diet [66]. Further studies with larger patient materials and longer follow-up time are needed to confirm, if these dietary interventions were beneficial, and could be recommended. In MNGIE, correlation between plasma thymidine levels and the severity of the phenotype has been observed [67]. Therefore, attempts to reduce the circulating nucleotide levels could result in disease improvement. Enzyme replacement therapy has been applied for MNGIE: infusion of platelets from healthy donors to patients with MNGIE reduced their circulating thymidine and deoxyuracile levels, and partially restored TP activity. The limitation of this therapy was the short half-life of platelets [68]. Allogenic stem cell transfusions have been given to two patients with MNGIE [69]. Although more experience is needed to illustrate the clinical benefit of that treatment, it opens up a possibility of treatment for disorders of the nucleoside metabolism. In MNGIE, also continuous ambulatory peritoneal dialysis has been used to reduce the thymidine levels, and this resulted in improvement of the symptoms during 3-year follow-up time [70]. Good animal models will enable testing these hypotheses *in vivo*.

6. Conclusive remarks

The diagnostic process in nuclear disorders of oxidative metabolism is not too different from that employed for other diseases and includes patient and family history, physical and neurologic examination, routine and special laboratory tests, muscle biopsy for morphology and biochemistry, and molecular genetics screening [71]. A mitochondrial disease manifesting at or soon after birth is more likely to be associated with nDNA than with mtDNA mutations, but until very recently, our profound ignorance regarding the mechanisms underlying mitochondrial gene transcription and translation and the complex interaction between the ''2 genomes'' has limited our diagnostic power. Mitochondrial DNA deletion and depletion syndromes, and disturbances in the mitochondrial translation machinery have become an increasingly important cause of a wide spectrum of infantile and childhood-onset multisystem disorders. Depletion syndromes could result from any imbalance of the mitochondrial dNTPs pools available for mtDNA replication, as well as abnormalities in either the mitochondrial helicase or DNA polymerase. Consistent with the different phenotypes, mtDNA depletion may affect specific tissue (most commonly, brain and muscle or liver) or multiple organs, including the heart and the kidney. Predictably, affected tissues show paucity of mtDNA-encoded translation products and multiple respiratory chain defects. More than 75% of these patients had onset during the first year of life, and the disease was rapidly fatal in most cases [3,72,]. Moreover, though the components of the complicated mitochondrial protein-synthesis machinery are exclusively nuclear encoded, the majority of mutation affects correct translation of mtDNA-encoded subunits of the OXPHOS system and accounts for a still undetermined number of genetic defects. Indeed, there is still limited information on the many mitoribosomal proteins; the several tRNA maturation enzymes; the aminoacyl-tRNA synthetases; the translation initiation, elongation, and termination factors; and the predictably larger number of unidentified factors needed for ribosome assembly [43,73].

The increasing number of nuclear governed mitochondrial diseases and its associated genes continues to increase the diversity of the genetic and clinical phenotypic heterogeneity of this group of disorders. Identifying the causative genes is not only important for adequate genetic counseling and prenatal diagnosis but also to have a better understanding of the disease pathophysiology leading to better therapy options. The increasing number of genes involved is a driving force for the development of high throughput strategies. The recent advances on sequencing technology will facilitate the molecular investigations of genes associated with mtDNA disorders in general. Reports concerning the use of next generation sequencing for the diagnosis of mitochondrial disorders are emerging [74,75,76]. In a recent report the use of target NGS for mitochondrial disorders proved its efficiency in clinical diagnosis as for 55% of the studied patients a clear molecular etiology was found. As more studies are reported the importance of applying this technology will be highlighted.

The problems faced by patients with mitochondrial respiratory chain disease are particularly severe. Diagnosis is difficult, treatment is largely ineffective, genetic counseling and prenatal diagnoses are uncertain or unavailable and the prognosis is unpredictable. Because diagnosis is imperfect and laborious, many patients undergo a whole battery of unnecessary investigations during the diagnostic process. Accurate focused diagnosis will save time, money and distress. Only by understanding the molecular genetic basis of these disorders, whether nuclear or mitochondrial, will any progress be made. Furthermore this will help patients, but will also lead to fundamental advances in our understanding of mitochondrial biology. Identification of new disease-causing gene(s) will hopefully provide insights towards novel therapeutic strategies.

Chapter highlights

- The chapter focus on diseases of intergenomic communication disorders mainly the ones involved in mtDNA integrity and mitochondrial protein synthesis
- Disorders affecting mtDNA stability lead to multiple deletions or depletion of mtDNA
- This group of disorders can affect a variety of organ with variable ages of onset
- *POLG* is frequently mutated being a hotspot for mitochondrial disease

- Diagnosis is difficult and laborious due to the increasing number of genes involved
- Therapy in mainly palliative however novel strategies are emerging
- Due to the increasing number of genes involved novel diagnostic strategies are emerging to optimize the diagnosis offered to these families

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Letter to the Editor

MPV17: Fatal hepatocerebral presentation in a Brazilian infant

The hepatocerebral forms of mtDNA depletion syndromes typically manifest soon after birth and cause premature death in childhood [1]. Presentation is usually with early liver failure, followed by development delay and muscle weakness during the first year of life, as well as lactic acidosis and hypoglycemia [2]. These conditions are associated with mutations in at least five genes [3] though further heterogeneity is expected. An ample array of *MPV17* mutations (Fig. 1) has been described in about 30 patients from different ethnicities.

We describe a Brazilian infant, the second child of apparently unrelated healthy parents, who presented neonatal hypoglycemia, digestive bleeding and sepsis in the first week of life. Laboratory tests also showed elevated levels of total and free serum bilirubin and transaminases. Amino acids were normal whereas organic acids revealed the presence of lactic acid (3× normal values) and Krebs cycle intermediates at age 2 months. Shortly after the patient manifested failure to thrive, severe hypotonia, nystagmus, poor echoarchitecture at liver ultrasound, and cirrhotic ascites. He died at 5-months-old with hepatic insufficiency associated with bleeding and multiple organ failure. He had severe mtDNA depletion (residual mtDNA levels were 2% in liver and 23% in muscle) and molecular studies detected a homozygous mutation c.186+2T>C in *MPV17*, a variant already reported with poor prognosis in a singleton [4]. The c.186+2T>C was also detected in the proband's elder sister who had presented at birth with neonatal hypoglycemia, failure to thrive and hepatic insufficiency, later developed seizures and hypotonia, and died at 10 months. The mutation was heterozygous in the parents who were from nearby villages in south Brazil.

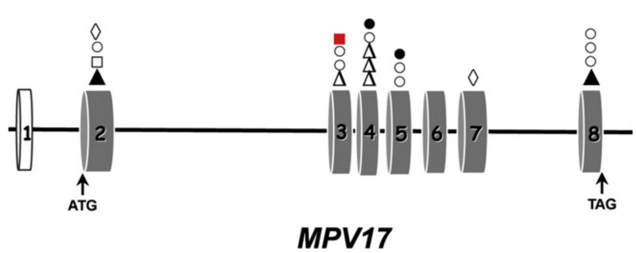

Fig. 1. Described mutations in the *MPV17* gene. ○ Missense mutation; ● Nonsense mutation; △ Small deletion; ▲ Gross deletion; ◊ Small insertion; □ Splicing mutation; ■ c.186+2T>C.

Our study expands the ethnic background of *MPV17*-mutated patients and will be important for an accurate genetic counseling and a prenatal diagnosis to the affected family. MtDNA depletion should be looked for in neonates with progressive cholestasis and neurological deterioration.

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SHORT COMMUNICATION

Novel *TTC19* mutation in a family with severe psychiatric manifestations and complex III deficiency

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Abstract Complex III of the mitochondrial respiratory chain (CIII) catalyzes transfer of electrons from reduced coenzyme Q to cytochrome c. Low biochemical activity of CIII is not a frequent etiology in disorders of oxidative metabolism and is genetically heterogeneous. Recently, mutations in the human tetratricopeptide 19 gene (*TTC19*) have been involved in the etiology of CIII deficiency through impaired assembly of the holocomplex. We investigated a consanguineous Portuguese family where four siblings had reduced enzymatic activity of CIII in muscle and harbored a novel homozygous mutation in *TTC19*. The clinical phenotype in the four sibs was consistent with severe olivo–ponto–cerebellar atrophy, although their age at onset differed slightly. Interestingly, three patients also presented progressive psychosis. The mutation resulted in almost complete absence of TTC19 protein, defective assembly of CIII in muscle, and enhanced production of reactive oxygen species in cultured skin fibroblasts. Our findings add to the array of mutations in *TTC19*, corroborate the notion of genotype/phenotype variability in mitochondrial encephalomyopathies even within a single family, and indicate that psychiatric manifestations are a further presentation of low CIII.

Célia Nogueira and José Barros contributed equally to this work.

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Keywords Complex III · *TTC19* · Cerebellar ataxia · Olivo–ponto–cerebellar atrophy · Psychiatric manifestations

Introduction

The autosomal recessive cerebellar ataxias (ARCAs) are a heterogeneous group of neurodegenerative disorders which can be classified according to their clinical and genetic traits [1, 2]. The possible etiologies encompass metabolic alterations in cellular organelles, including lysosomes, peroxisomes, and mitochondria, and deficiency of DNA repair and chaperone activities [2]. In this field, the development of traditional and more sophisticated technical approaches, including exome sequencing [3], is leading to the identification of a growing number of causative genes and new disease entities [1].

Ubiquinol–cytochrome *c* reductase (complex III, E.C.1.10.2.2) (CIII) is the third component of the mitochondrial respiratory chain (RC), and it catalyzes the transfer of electrons from reduced coenzyme Q to cytochrome *c* with a concomitant translocation of protons across the inner membrane [4]. CIII is made up of 11 subunits [5], only one of which (cytochrome b) is encoded by mtDNA; the remaining proteins are encoded by nuclear DNA genes. These include cytochrome c1, the Rieske iron–sulfur protein (RISP), two "core" subunits (Core 1 and Core 2), and six additional, smaller proteins (UQCR6-11), whose functions are still largely unknown [5, 6]. Low or very low biochemical activities of CIII [MIM 124000] are a relatively rare cause of dysfunction of oxidative metabolism [7]. Mutations in *MT-CYB* and in the assembly factor *BCS1L* account for the vast majority of cases with CIII deficiency [8]. Less frequently, mutations in mtDNA-encoded tRNAs, or single large-scale mtDNA deletions, may also account for a partial reduction of the biochemical activity of CIII [8].

Recently, mutations in the human tetratricopeptide 19 gene (*TTC19*), which encodes a still poorly characterized protein thought to be important in early CIII assembly, have been described in association with young-onset neurodegeneration in two unrelated Italian kindred as well as adult-onset, rapidly progressing encephalopathy in an unrelated case [5].

We here report four patients from a family found to harbor a novel *TTC19* mutation. Whilst all the patients presented similarly reduced CIII enzymatic activity in muscle and a comparable pattern on brain neuroimaging, the onset, clinical presentation, and course of their cerebellar ataxia and psychiatric dysfunctions varied significantly.

Patients and methods

In a Portuguese kinship (Fig. 1a), four sibs (three men and one woman) were affected. Although formal consanguinity was denied, both sets of grandparents originated from neighboring villages in a relatively isolated area in northern Portugal. The recruitment and study protocol was approved by the medical research ethics committee of our institutes. The participating family members in this study underwent a thorough neurological examination. In addition, the following workup was performed: brain and spinal MRI, complete blood count and metabolic screen, EMG and nerve conduction studies, audiometric examination, and brainstem auditory, visual, motor, and somatosensory evoked potentials.

RC complex activities in skeletal muscle homogenate and cultured skin fibroblasts were measured using an existing spectrophotometric method [9]. Human fibroblasts were obtained from diagnostic skin biopsies and grown in regular DMEM or in glucose-free medium supplemented with 5 mM galactose to induce a stress culture condition, as reported [10], with or without the following additives: 0.25 mM AICAR (Tocris, Bioscience, Bristol, UK), 400 μM bezafibrate (Sigma-Aldrich, St. Louis, MO), and 1 mM H_20_2 (Sigma-Aldrich). Cells were analyzed 72 h post-treatment for rate of growth and viability, total ATP production, and amount of reactive oxygen species (ROS). Cellular ATP content and viability were evaluated using the CellTiter-Glo® (Promega, Madison, WI) system, according to the manufacturers. For intracellular ROS production, cells (2.5×10^5) were incubated with 5 μM CM-H_2DCFDA (Molecular Probes, Invitrogen, Paisley, UK) in 1 ml buffer (10 mM HEPES, 142 mM NaCl, 2 mM KCl, 1.2 mM KH_2PO_4, 1 mM $MgSO_4$, 1.3 mM $CaCl_2$, pH 7.4) supplemented with 1 % FBS for 15 min at 37 °C. After washing with the same buffer, the cells were incubated for 30 min with 1 mM H_2O_2 in 1 ml buffer. Fluorescence was measured at excitation and emission wavelengths of 490 and 525 nm, respectively, using a Cary Eclipse Fluorescence Spectrophotometer (Agilent, Santa Clara, CA).

Blue native gel electrophoresis (BNGE) and 2D SDS-PAGE studies in patient II-06 and control tissues were performed essentially as described elsewhere [11, 12]. For SDS-PAGE and immunofluorescence analyses, we used methodologies familiar to our laboratories [10], employing the following monoclonal antibodies: complex I—39-kDa subunit (NDUFA9), complex II—70-kDa subunit (SDHA), complex III—Core 2; complex IV—subunit II (CIV-II), anti-porin (all from Mitosciences, Eugene, OR), and a polyclonal antibody anti-TTC19 (Sigma-Aldrich).

Genomic DNA was purified from blood using standard procedures. The coding exons and exon–intron boundaries of *TTC19* [NG_029806.1] were sequenced using the BigDye v3.1 chemistry (Applied Biosystems, Foster City, CA). MtDNA copy number in muscle was investigated by a real-time qPCR assay [13] and results compared to levels seen in tissue from age-matched normal controls. Total RNA was isolated from cultured fibroblasts (RNeasy Mini Kit, QIAGEN, Hombrechtikon, Switzerland) from the

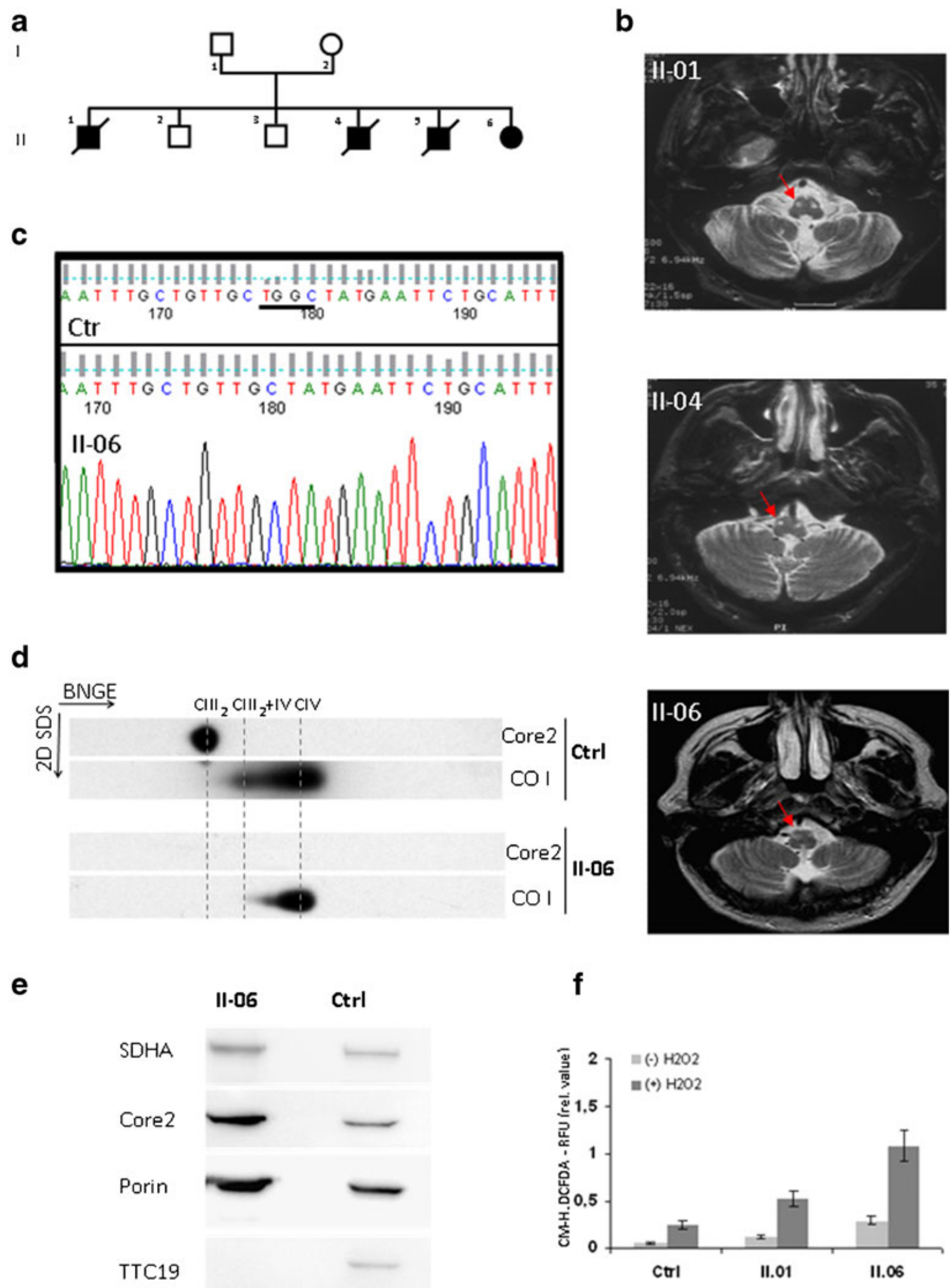

Fig. 1 Family pedigree, brain MRI, and molecular studies in the new Portuguese kindred harboring a mutation in *TTC19*. **a** Family tree. *Square* men, *circle* female, *filled symbols* affected individuals. *Slash* indicates deceased subjects. **b** Brain MRI in three different affected patients (II-01, II-04, II-06) in this family. *Arrows* indicate pontine atrophy. **c** Electropherograms of the *TTC19* gene flanking the homozygous c.962_967delTGGC (p.A321Afs*8) variant identified in patient II-06. Wild-type sequence in a control (*Ctr*) is also shown. The deleted bases are *underlined*. **d** BNGE followed by 2D SDS-PAGE in muscle homogenates from patient II-06 and a control (*Ctrl*) using antibodies against the Core 2 subunit to detect complex III and subunit I of cytochrome *c* oxidase (CO I) for complex IV. **e** A representative immunoblot analysis of muscle homogenates from control (*Ctrl*) and patient II-06 using specific antibodies against subunits of complex II (SDHA), complex III (Core 2), and TTC19. VDAC/porin was used to control for equal loading. Similar results were seen in cultured skin fibroblasts. **f** ROS production measured by CM-H_2DCFDA and expressed as relative fluorescent units (*RFU*) in skin fibroblasts from control (*Ctrl*) and patients II-01 and II-06 incubated with or without with 1 mM H_2O_2 for 30 min. Data are mean ± SD of five different determinations. Significance was set at $p<0.05$

patient prior to cDNA synthesis (performed using a first-strand cDNA synthesis kit [Promega, Dübendorf, Switzerland]).

Results

Case II-01, the propositus (Fig. 1a), was a 49-year-old man who had no clinically relevant complaints until the age of 27 years, when he manifested progressive back pain, clumsiness, gait difficulties, and a generalized anxiety disorder. Over the following years, additional psychiatric manifestations appeared, including major depression and visual hallucinations which did not respond to antipsychotic drugs. Neurological examination at age 37 revealed difficulties in tandem gait; loss of balance while climbing stairs; segmental ataxia, observable as dysmetria in the nose–finger and heel–shin tests; and interruptions in fast alternating hand movements. Dysarthric speech, dysphagia, and slow saccades were also detected. Subsequently, the patient displayed moderate spastic paraparesis with pyramidal tract signs affecting both the upper and the lower limbs. By the age of 42, he presented severe muscle atrophy and, unable to walk or stand, was bedridden. His psychosis had worsened as a result of the onset of visual and acoustic hallucinations. He died of respiratory insufficiency aged 49 years.

Three additional sibs (II-04, II-05, and II-06) showed an ataxic syndrome and psychiatric manifestations (see clinical appendix for details). The parents and two additional siblings (in their 50s), who were all asymptomatic, had no major neuropsychiatric complaints and a normal examination. Table 1 summarizes relevant data in this family.

Brain imaging in all the patients at different ages showed a pattern of olivo–ponto–cerebellar atrophy (Fig. 1b) and abnormal hypersignal in the caudate, putamen, medullary olives, cerebellar dentate nucleus, and medial midbrain on T2-weighted MRI scans. Cortical brain atrophy (especially in the frontal lobe) appeared at a later age in patients II-01 and II-04. No white matter changes or structural brain lesions were observed.

Routine histochemical stains for oxidative metabolism in muscle biopsies were unremarkable in the four patients, whereas measurement of RC enzymes revealed a marked reduction of CIII activity in all of them (on average, residual activity corresponded to 33 % of that recorded in age-matched normal controls) (Table 1).

We identified a novel c.963_966delTGGC mutation in *TTC19* (Fig. 1c) in the propositus and three sibs. This mutation predicts a frameshift and the synthesis of a truncated protein (p.A321Afs*8), lacking about 35 % of its carboxy terminus. The new mutation was found to be homozygous in the four patients, heterozygous in their parents and in unaffected sibs, and absent in 300 ethnically matched controls. The c.963_966delTGGC was expressed in tissues from patients II-01 and II-06, and the amount of mRNA/*TTC19* transcript appeared to be semiquantitatively reduced in muscle from case II-06 (not shown). Interestingly, we also observed a moderate reduction of mtDNA copy number in II-06 (residual mtDNA levels were, on average, 45 % of those of controls) (supplementary Fig. 1). Using Western blotting, we found no protein using a specific anti-TTC19 antibody in muscle and fibroblasts from patients II-01 and II-06 (Fig. 1d). The immunodetection pattern of the RC complexes was within normal values in skin fibroblasts (supplementary Fig. 1). Using BNGE followed by 2D-SDS and anti-Core 2 antibody, there were nearly absent spots for CIII and $CIII_2$ + IV supercomplexes in muscle from patient II-06 compared to control muscle (Fig. 1e), suggesting early impairment of assembly of CIII, as already outlined elsewhere [8]. A less profound defect was seen in skin fibroblasts (supplementary Fig. 1).

ATP production in skin fibroblasts was not reduced in either glucose or galactose medium (supplementary Fig. 1). We also observed a statistically significant increase ($p<0.03$) of ROS production in basal condition and after short-term H_2O_2 treatment (Fig. 1f). Neither cellular ATP level nor ROS production was influenced by treatment with AICAR or bezafibrate, drugs known to enhance cellular oxidative metabolism (not shown).

Discussion

More than 20 different ARCAs are currently documented, and among these, the rare mitochondrial forms are increasingly being recognized. The best known are Friedreich ataxia and mitochondrial recessive ataxic syndrome (with mutations in *POLG*), two progressive diseases, but more static conditions are also described [2]. We reported herein an additional form of mitochondrial ARCA in a family where four siblings presented markedly reduced biochemical activities of CIII associated with overt psychiatric illness. The psychiatric component was less obvious in previously reported cases of mutations in *TTC19*, although two of those patients lacked motivation and displayed avoidance behavior and poor social interaction [5].

Psychiatric manifestations are generally uncommon in ARCAs [14] and more often seen in dominant forms, such as SCA7 and SCA8 [15]. The psychotic manifestations seen in these Portuguese *TTC19* patients are also reminiscent of the severe form of SCA17, a dominant “cerebello–olivary” syndrome with abnormal repeat expansions in the *TBP* gene. It is noteworthy that *TBP* has a role in the regulation of gene expression [16] and impacts on chaperone protein response [17]. This is also true of several members of the TPR family of proteins [18] and possibly also of TTC19 [5]. However, given the likelihood of inbreeding in the family we described, we cannot completely rule out the existence of additional mutations responsible for the severe psychiatric syndrome observed.

This report prompts two main considerations. First, the Portuguese family we described is additional evidence that it is difficult to predict the onset (timing and symptoms) and progression of a neurodegenerative disease due to defective oxidative metabolism [8–10] even when multiple cases have already occurred. Mutations in *TTC19* were initially linked to severe neurodegenerative disorders in childhood and in adult cases [5]. In the family described herein, the age at onset and pattern of progression varied significantly, with II-04 and II-05 manifesting symptoms in their teens and showing a rapid downhill course, and II-01 and II-06 showing a later onset with a progressive disease course. In II-06, neuropsychiatric features appeared in spite of annual monitoring and attempts to treat the symptoms. Reason for such variety remains unknown, but it could be related to levels of biochemical defects in specific brain areas or even to secondary alterations in the mtDNA. The latter is thought to contribute to a number of neurodegenerative diseases of

Table 1 Clinical and laboratory findings in patients harboring mutations in *TTC19*

		Cases reported elsewhere [5]				This work			
		Family 1		Family 2	Family 3	II-01	II-04	II-05	II-06
Ethnic origin		Italian		Italian	Italian	Portuguese			
Age at onset (years)/sex		5/F	10/M	5/F	43/M	27/M	12/M	15/M	34/F
Neurological examination	Signs/symptoms at onset	Learning disability, gait ataxia	Learning disability, gait ataxia	Regression of language, gait ataxia	Weakness, extrapyram.	Mood disorder, gait ataxia	Compulsive lying	Aggressive behavior	Avoidance behavior
	Functional status	Wheelchair bound (37 years)	Bedridden (26 years)	Bedridden (19 years)	Deceased (45 years)	Deceased (49 years)	Deceased (33 years)	Deceased (30 years)	Gait ataxia
	Gait ataxia	+	+	+	+	+	+	+	+
	Nystagmus	+	+	na	na	na	na	na	na
	Dysphagia/dysphonia	+	+	na	na	+	+	+	+
	Dysarthria	+	+	+	+	+	+	+	+
	Dysmetria	+	+	+	+	+	+	+	+
	Spasticity	na	na	na	+	+	+	+	na
	Hemiparesis	+	na	na	na	+	na	+	na
	Enhanced tendon reflexes	+	na	na	na	+	+	+	+
	Babinski sign	+	na	na	na	na	+	+	+
	Dystonic postures/ extrapyramidal features	na	+	+	+	na	na	+	na
	Muscle weakness	+	na	na	+	+	+	+	–
	Ptosis, optic atrophy, hearing loss	na	+	na	na	na	na	na	na
	Other features	na	PEG, tracheostomy	Scoliosis, cardiorespiratory arrest	Low frontoparietal perfusion (SPECT)	na	na	na	na
Cognitive/ behavioral	Cognitive decline	na	+	na	na	+	+	+	+
	Major depression/ psychosis	na	na	na	na	+	+	+	+
	Psychiatric disturbances	na	na	na	na	+	+	+	+
	Lack of initiative	na	na	na	+	+	+	+	+
	Poor social interactions/ avoidance behavior	na	na	+	+	+	+	+	+
Neurophysiology	EMG	Neurogenic	na	na	Neurogenic	Neurogenic + myopathic	Neurogenic + myopathic	na	Neurogenic + myopathic
	NCV/Axonal neuropathy	na	na	+	+	+	na	+	na
Brain MRI features	Leukoencephalopathy	+	–	–	–	–	–	–	–
	Hyperintense caudate nucleus	+	na	na	na	+	+	+	na
	Cerebellar atrophy	+	na	+	na	+	+	+	+

Table 1 (continued)

		Cases reported elsewhere [5]				This work			
		Family 1		Family 2	Family 3	II-01	II-04	II-05	II-06
	Pontine atrophy	+	na	+	na	+	+	+	+
	Inferior olives	+	na	+	na	+	+	+	+
	Substantia nigra	+	na	na	na	na	na	na	na
	Putamen	+	na	na	+	na	+	+	na
	Caudate nuclei	+	na	na	+	na	+	+	na
	Medulla oblongata	na	na	+	na	na	na	na	na
	Mesencephalon	na	na	+	na	na	+	na	na
	Cortical atrophy	na	na	na	+	+	+	+	−
	^{1}H-MRS	Lactate peak	na	na	Lactate peak	na	na	na	na
Metabolic data	High lactate (blood/CSF)	−	na	+ (CSF)	+ (blood)	+ (blood)	na	na	na
Muscle biopsy	Morphology	Normal	na	Normal	Lipid accumulation, few COX negative fibers	Normal	Normal	Normal	Normal
	Residual activity complex III	19 %	14 %	17 %	8 %	30 %	31 %	33 %	39 %
Skin fibroblasts	Residual activity complex III	77 %	81 %	89 %	na	45 %	na	na	83 %

M male, *F* female, *na* no data available, *PEG* percutaneous endoscopic gastrostomy, *EMG* electromyography, *NCV* nerve conduction velocities, *SPECT* single-photon emission computed tomography, *COX* cytochrome *c* oxidase, + present, − not present adult life [19]. Also, occurrence of psychiatric manifestations is rather unusual in disorders of oxidative metabolism, though comorbidity between mitochondrial disorders and psychiatric illness has been described as the presenting features in fewer patients [20]. Second, our data add to the relatively limited array of variants in genes related to CIII deficiency. Like the previously described *TTC19* mutation, the new c.963_966delTGGC seems to lead to a significant reduction of CIII holoenzyme and impairment of early assembly steps, or overall stability, of the holoenzyme. However, the mechanism by which lack of TTC19 and the ensuing biochemical features affect cortical and deep brain neurons and lead to the clinical manifestations seen in our family remains largely unexplained. It is tempting to hypothesize that the high levels of ROS production seen in cells from patient II-06—and in II-01, though at a lesser extent—are even higher in selective brain structures probably because of the lack of functional components of CIII holoenzyme (for example, RISP) that are usually assembled in later stages [21]. Interestingly, severe ROS damage, impaired motor coordination, and neurodegeneration in limbic system structures have been observed upon ablation of RISP in a brain-specific mouse model of CIII deficiency [22].

To summarize, we here described the first family outside Italy with a mutation in *TTC19*. The novel change manifested as a disorder characterized by degeneration of olivary–cerebellar neurons and a progressive psychiatric syndrome, findings that should alert clinicians while searching for additional cases.

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Conflict of interest The authors declare that they have no conflict of interest.

Appendix—clinical data in patients harboring a new mutation in *TTC19*

Case II-04 was a 33-year-old man who had displayed obsessive–compulsive behavior since his teens. At the age of 24, he was hospitalized and found, on psychiatric examination, to be a healthy young man who displayed childish behavior and frequently used "lies to communicate." The patient, at that time, also complained of generalized anxiety and a sleep disorder. Medical records also contained reports of episodes of mysticism and visual hallucinations (the patient often said "I can see dead people talking to me"). His neurological examination was unremarkable. A few years later, his clinical profile also included abulia and bradypsychism with episodes of confusion and spatial disorientation. A neurological examination performed during hospitalization at the age of 27 years revealed mild cerebellar ataxia with dysarthria, pyramidal tract signs in the lower limbs (leg stiffness, enhanced deep tendon reflexes, bilateral Babinski sign), and generalized muscle atrophy and wasting. The cranial nerves and peripheral nervous system were spared. The patient's disease progressed rapidly; he became mutacic and cachectic, and died at the age of 33 of respiratory insufficiency.

Case II-05 was a 30-year-old man who was prone to violent behavior during his teens and had physically attacked an officer during his compulsory military service at age 18. Once expelled from the army, he was committed to a psychiatric unit until the age of 22 years. When he was 23, he was reported for domestic violence and aggression in the workplace. Medical records from that time describe a mutacic patient with mild cognitive impairment and a combination of depressive and psychotic symptoms, as well as dysphagia and dysarthria. Neurological examination also revealed the presence of "primitive" reflexes (sucking, palmomental, and grasping), ataxic gait with bilateral pyramidal syndrome, and extrapyramidal features (bradykinesia and hand dystonia). He died of cardiac arrest aged 30 years.

Case II-06 is a 38-year-old woman who had no significant medical complaints until the age of 34. In view of her three older brothers' clinical symptoms, she had been carefully monitored since the age of 15 years and her parents had reported only mild clumsiness and swerving while riding a bike. At the age of 34, she began to display rude manners, compulsory polyphagia, a major mood disorder, and auditory hallucinations (often saying that dead relatives were inciting her to commit suicide). Neurological examination at this time revealed gait ataxia, bilateral finger–nose dysmetria, and inability to perform tandem gait. Neuropsychological assessment revealed significant impairment of attention, psychomotor speed and language, as well as ideomotor apraxia and memory loss. There were also clinical features suggestive of pyramidal and extrapyramidal tract dysfunction. Treatment with idebenone and haloperidol was attempted without success.

All the patients were, at some time during the disease course, screened for genetic alterations associated with Friedreich ataxia, SCA1, SCA2, MJD1/SCA3 (Machado–Joseph disease), SCA6, SCA7, SCA12, SCA17, dentatorubropallidoluysian atrophy, and FXTAS. Screening of the POLG, APTX, and SETX genes was negative in patient II-01, as were whole mtDNA sequencing and screening of the genes encoding the structural component of CIII (II-01 and II-04) and BCS1L (II-01 and II-06).

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Letter to the Editor

Identification of maternal uniparental isodisomy of chromosome 10 in a patient with mitochondrial DNA depletion syndrome

Keywords:
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Twinkle

Twinkle, the mitochondrial helicase encoded by *C10orf2*, serves a key function in mtDNA replication [1] and its mutations (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=C10orf2) associate with a broad spectrum of clinical conditions characterized by qualitative or quantitative defects of mtDNA, including infantile-onset spinocerebellar ataxia, progressive external ophthalmoplegia, and the syndrome of hepatocerebral mtDNA depletion (MDS) [2].

We studied a Portuguese child, born to unrelated healthy parents, who presented hypotonia and Pierre-Robin sequence (MIM 311895) with mandibular retrognathism, partial syndactyly of 2nd–3rd fingers, bilateral strabismus, and eyelid asymmetry. The child manifested severe intellectual and developmental disability and nystagmus in the following months. Brain MRI revealed brain atrophy and a thin corpus callosum (Fig. 1A). Serum lactate levels were increased, OXPHOS activities in a skeletal muscle biopsy were in the low normal range and there was mtDNA depletion in muscle (~80% normal age matched controls).

In the patient we identified two "in cis" homozygous splice site mutations in *C10orf2* ([c.1593-3T>C + c.1593-5C>T]; Fig. 1B) predicted *in silico* to activate a new cryptic acceptor site and lead to missplicing. The novel mutations were heterozygous in the mother and the healthy brother whereas the father was wild-type. Once we had ruled out false paternity and a multi-exon deletion on the paternal allele by MLPA, we genotyped three microsatellite markers and 12 SNPs flanking *C10orf2* on chromosome 10q24. Whilst a number of markers were either uninformative, SNP genotyping was also consistent with segmental uniparental isodisomic transmission (UPD) of the maternal chromosome 10 (Fig. 1C). A similar condition has already been reported for *TYMP* and *DGUOK* [3,4] two other causes of MDS.

Description of UDP in a patient presenting mutations in Twinkle corroborates the need of a full molecular examination of apparently homozygous changes in MDS to define more precisely the risk of recurrence in subsequent pregnancies.

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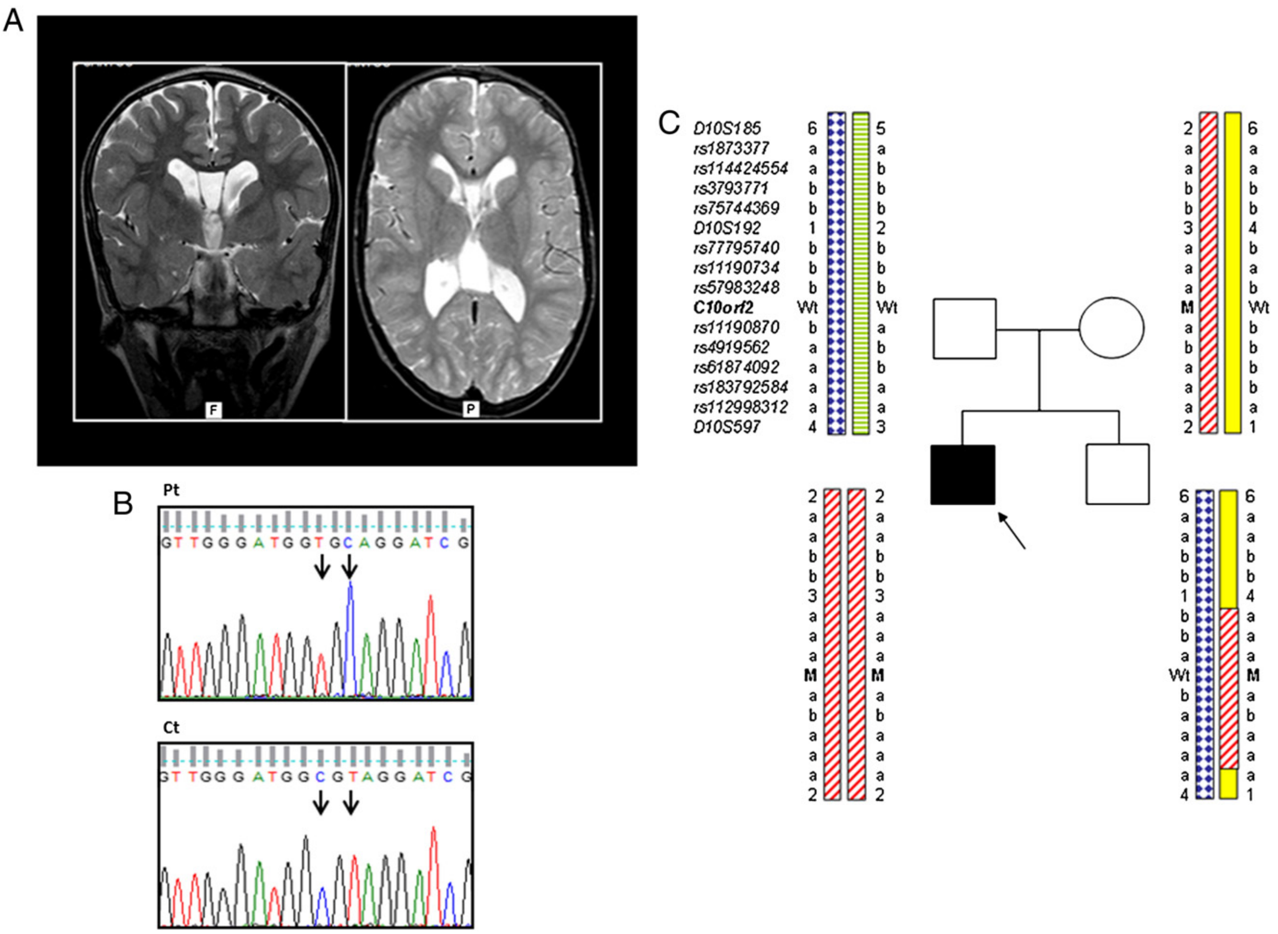

Fig. 1. Neuroimaging and molecular studies in the patient harboring a maternal isodisomic inheritance of chromosome 10. A) Brain MRI (coronal and axial images). There was decreased intensity in the posterior periventricular white matter and a thinner corpus callosum. No pathological signal changes were seen in the striatum, brainstem and dentate nuclei. B) Electropherograms of the *C10orf2* gene flanking the homozygous splice site mutations ([c.1593-3T>C + c.1593-5C>T] identified in the patient (Pt). Wild-type sequence in a control (Ct) is also shown. The mutated bases are indicated with an arrow. C) Segregation analysis of polymorphic microsatellite markers and single nucleotide polymorphisms flanking the *C10orf2* gene suggested maternal UDP. Family tree: square, men; circle, women; filled symbol is the affected patient (arrow). M, *C10orf2* mutated sequences; Wt, wild-type sequence.

A novel *SUCLA2* mutation in a Portuguese child associated with "mild" methylmalonic aciduria

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Abstract

Succinyl-CoA synthase is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinate and adenosine triphosphate (ATP) from succinyl-CoA and adenosine diphosphate (ADP) in the tricarboxylic acid cycle. This enzyme is made up of an α and β subunits encoded by *SUCLG1* and *SUCLA2*, respectively. We present a child with severe muscular hypotonia, dystonia, failure to thrive, sensorineural deafness and dysmorphism. Metabolic investigations disclosed hyperlactacidemia, moderate urinary excretion of methylmalonic acid and elevated levels of C4-dicarboxylic carnitine in blood. We identified a novel homozygous p.M329V in *SUCLA2.* In cultured cells, the p.M329V resulted in a reduced amount of the SUCLA2 protein, impaired production of mitochondrial ATP, and enhanced production of reactive oxygen species (ROS) which was partially reduced by using 5-aminoimidazole-4-carboxamide ribonucleotide - AICAR - in the culture medium. Expanding the array of *SUCLA2* mutations, we suggested that ROS scavengers have the likelihood to impact on disease prognosis.

Keywords

SUCLA2; Methylmalonic aciduria; Mitochondrial DNA depletion; Encephalomyopathy; Succinate-CoA ligase

Introduction

The encephalomyopathic form of infantile mitochondrial depletion syndrome associated with methylmalonic aciduria (MIM 612073) has been linked to mutations in *SUCLA2* and in *SUCLG1*, the genes coding for the β and α subunits of the ADP-dependent isoform of succinyl-CoA synthase (SCS) (EC 6.2.1.5), respectively. SCS is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinyl-CoA from succinate and coenzyme A (CoA). The reverse reaction occurs in the Krebs cycle, while the forward reaction may produce succinyl-CoA for activation of ketone bodies and heme synthesis. SCS is made of an invariant α subunit encoded by *SUCLG1* and a β subunit that determines the enzymatic nucleotide specificity. The Adenosine triphosphate (ATP)-specific β subunit encoded by *SUCLA2* catalyzes ATP dependent reactions, and the Guanosine triphosphate (GTP)-specific β subunit encoded by *SUCLG2* is a component of the second SCS isoform (EC 6.2.1.4) which catalyzes guanosine diphosphate-dependent reactions.[1-4]

The SUCLA2 protein is mostly expressed in brain, skeletal muscle, and heart and its pattern of expression may be related to the high energy demand of those catabolic tissues.[5,6] Several studies have suggested that SUCLA2 forms a complex with nucleoside diphosphate kinase (NDPK), that is important for the salvage of deoxyribonucleotides for mitochondrial DNA (mtDNA) synthesis and directly implicate the GTP-specific β subunit in control of mtDNA copy number.[6]

A “mildly” elevated urinary methylmalonic acid (MMA) in combination with a characteristic metabolite profile (that is, elevated methylcitrate, lactate, carnitine esters, 3-hydroxyisovaleric acid) represent useful “red flag” for a SCS disorder. Other metabolic findings usually consist of elevated C4-dicarboxylic carnitine in blood, elevated plasma MMA and high lactate concentration. Patients with low enzyme activity usually die as infants (often suddenly), but some of them have a longer survival and present clinical features suggestive of mitochondrial encephalomyopathy, including psychomotor delay and, almost invariably, progressive dystonia and hearing loss.[7] To date, 32 individuals harboring mutations in *SUCLA2* have been described often presenting combined multiple deficiencies of the mitochondrial respiratory chain.[8]

We here report an additional patient carrying a novel mutation in *SUCLA2* in association with progressive failure to thrive, muscle weakness and deafness. We also investigated in primary cells the functional consequences of the mutation.

Case Report

Clinical and metabolic investigations were performed in a 2-year-old child presenting since birth with facial dysmorphism, severe muscular hypotonia with muscular atrophy and failure to thrive. This is the first child born to healthy, unrelated parents. Pregnancy and delivery were uneventful. Apgar scores were 9 and 10 at 1 and 5 minutes, respectively. Birth weight was 3kg, height 50 cm, and head circumference was 34.5 cm (50^{th} centile). Since birth the baby boy presented with hypotonia and frequent vomiting. Growth retardation and developmental delay were noticed since the age of 3 months. Initial investigations disclosed a male karyotype with normal thyroid, renal and hepatic functions, as well as normal blood lactate levels. Plasma amino acids and urinary organic acid profiles showed unspecific abnormalities. Cardiac ultrasound was normal whereas brain MRI disclosed slight cerebral atrophy and normal myelin pattern. The patient was later referred to the Hospital Pediátrico de Coimbra at age 16.5 months because of a possible inherited disorder of metabolism. At that age, his physical examination was significant for developmental delay, failure-to-thrive, microcephaly, muscular atrophy with dystonic posturing, recurrent vomiting and high ammonia level (115 µM, normal: 25-44µM) in blood.

Genomic DNA was purified from peripheral blood using standard procedures. The coding exons and exon-intron boundaries of *SUCLA2* [NM_003850.2] and *SUCLG1* [NM_003849.3] were sequenced using the BigDye v3.1 chemistry (Applied Biosystems, Foster City, CA) as described elsewhere.[4] Large scale, multi-exon deletions were analyzed by MLPA using commercially available SALSA MLPA P089 probemix (MRC-Holland, The Netherlands).

Human fibroblasts were obtained from a punch biopsy of skin and grown in regular Dulbecco's Modified Eagle's Medium (DMEM) medium unless otherwise stated. For SDS-PAGE analyses of cultured cells, we used methodologies familial to our laboratories, and employed the following monoclonal antibodies: complex I-39 kDa subunit (1:500, NDUFA9); complex II-70 kDa subunit (1:10.000, SDHA); complex III (1:500, Core2); complex IV-subunit II (1:500, CoxII); complex V-subunit alpha (1:500, ATP α); anti-porin (1:5000). All antibodies were from MitoSciences (Abcam, Cambridge, MA). We also used a polyclonal anti-SUCLA2 antibody (1:1000, Abcam).[9]

For cellular studies, cultured skin fibroblasts were grown in DMEM or in glucose-free medium supplemented for 72 hrs with 5 mM galactose to induce a stress condition, as reported, with or without the following additives: 0.5 mM 5- aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Tocris, Bioscience, Bristol, UK) and 500 µM H_2O_2 (Sigma-Aldrich, St. Louis, MO).[10]

MtDNA copy number in fibroblasts was investigated by a described qPCR assay in real-time and results compared to the levels seen in cells obtained from five age and sex-matched controls.[11]

Cells (1×10^4), incubated in a 96-well plate, were analyzed 72h post-treatment for rate of reactive oxygen species (ROS). After washing with respiration buffer (142 mM NaCl, 10 mM HEPES, 2 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.4) supplemented with 1% fetal calf serum (FBS) (ROS buffer), we added 5 µM 5-(and-6)-chloromethyl-2,7-dichlorodihydro-flouorescein diacetate, acetyl ester (CM-H_2DCFDA) (Molecular Probes, Invitrogen, Paisley, UK) in 100 µl of ROS buffer just prior incubation (15 min at 37°C). After washing with the same buffer, cells were incubated 30 min at 37°C with 100 µl of ROS buffer with or without 500µM of H_2O_2. Plates were subsequently washed with the same buffer, 100 µl of ROS buffer were added and fluorescence detected. Fluorescence was measured at excitation and emission wavelengths of 490 nm and 527 nm, respectively, using a Varian Cary Eclipse Fluorescence plate reader (Agilent Technologies, Santa Clara, CA).

Total ATP levels were measured using the Luminescence ATP Detection Assay System (PerkinElmer Life Sciences, Waltham, MA). Briefly, 1 x 10^4 cells were seeded in a 96-well plate and incubated 48h in DMEM. Plates were then washed with ATP record buffer (156mM NaCl, 3mM KCl, 2 mM $MgSO_4$, 1.25mM KH_2PO_4, 2 mM $CaCl_2$, 20mM HEPES, pH 7.35) and incubated 2h in ATP record buffer with the following conditions: 10 mM glucose; 10 mM glucose plus 2.5 µg/ml oligomycin (glycolytic ATP generation) and 5 mM 2-deoxy-Dglucose plus 5 mM pyruvate (oxidative ATP production). Cells were incubated with the luciferin/luciferase reagent and the ATP production in samples was measured using an Orion L microplate luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany).[12]

Results

During hospitalization, metabolic workup in the proband revealed a compensated metabolic acidosis with persistently high serum lactate (2.2 to 9 mM, normal < 1.3), and elevated lactate/pyruvate ratio (>25, normal: <10). There were also fluctuating hyperammonemia (up to 75 µM), high alanine, proline and glycine levels in plasma, "mildly" elevated urinary MMA and significantly high urinary levels of Krebs cycle intermediates. Acylcarnitine profile in blood showed an elevated C4-dicarboxylic carnitine (C4DC). Brainstem auditory evoked potentials showed a pattern suggestive of moderate sensorineural hearing loss. Based on laboratory findings, the patient was put on a protein restricted diet, with nasogastric tube feeding and combined vitamins (B1, B2, B9 and coenzyme Q10 supplementation. At the age of 18 months, the child manifested axial hypotonia (no head control), severe generalized dystonia, muscle wasting and constipation. All anthropometric parameters were below the 5^{th} centile (weight 7.1 kg, height 76.8 cm, head circumference 45.2 cm) in spite of adequate caloric intake. Brain MRI scans showed worsening of cerebral atrophy and slightly abnormal signals in deep brain (Figure 1). At age 24 months, the child's global developmental quotient (DQ) was below 50 (< 1 centile), with a mental age of 6 months (using the Griffths scale.[13] Since parents refused to grant permission for a diagnostic muscle biopsy, the child underwent a punch skin biopsy with written parental consent.

Direct sequencing of *SUCLA2* in blood DNA revealed a homozygous c.985A>G/p.M329V in exon 8 (Figure 2A) whereas no mutations were seen in *SUCLG1* and *SUCLG2*. The parents were heterozygous carriers of the p.M329V mutation. The missense change was novel and not detected in a large set of in-house ethnically-matched control chromosomes and in dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/) and Exome Variant database (evs.gs.washington.edu/EVS/). The mutation seems predictably deleterious after assessments *in silico* with Condel (bg.upf.edu/condel/), SIFT (sift.jcvi.org/), Mutation Taster (www.mutationtaster.org/), and Polyphen 2 (genetics.bwh.harvard.edu/pph2/index.shtml) and affects an amino acid that is highly conserved in different species (Figure 2B). A moderate reduction of mtDNA copy number (on average, 69% of normal controls) was observed in cultured skin fibroblasts after replacing galactose for glucose in the cell medium. Western blotting showed a ~30% decrease of SUCLA2 protein in patient's fibroblasts (Figure 2C) with normally expressed components of the respiratory chain complexes. Total and mitochondrial ATP productions in skin fibroblasts were 22% and 50% ($p<0.03$), respectively when compared to normal control values (Figure 3A). We also observed a statistically significant increase of ROS production in basal condition and after short-term H_2O_2 treatment (Figure 3B, $p<0.05$). ROS production was restored to normal levels upon treatment with 0.5 mM AICAR, a compound known to enhance cellular oxidative metabolism.[10]

Discussion

This case offers at least two significant points for discussion. The clinical and biochemical phenotype of our patient is highly similar to previously reported children harboring mutations in *SUCLA2* and it represents the first evidence of a SCS disorder in Portugal, widening the ethnic and allelic presentation of the disease.[4] The novel mutation meets several canonical criteria for pathogenicity. First, it was homozygous in the child and heterozygous in the parents, but absent in polymorphic databases or in over 200 ethnically matched controls. Second, the mutation affects a residue highly conserved during evolution and it is associated with a reduced amount of protein in cultured primary cells. Third, the mutation affected mitochondrial production of ATP and resulted in low mtDNA abundance in cultured cells. Reduction of mtDNA copy number, ranging from 50% and 60% of control values, has been shown in previous patients carrying loss-of-function mutations in *SUCLA2*.[4] A higher reduction is expected to occur in skeletal muscle or brain. Our data further confirm that the combined biochemical profile outlined in the introduction is suggestive of SCS disease.

An additional observation arising from our study relies on the reduction on ROS production upon AICAR treatment in cells from a *SUCLA2* patient. AICAR has already been shown to improve growth and ATP content in cells harboring mutations in genes encoding subunits of the respiratory chain complexes I and IV and it is said to fight the pathological mechanisms in a mouse model of mitochondrial encephalomyopathy.[10,14] As a pharmacological activator of AMP activated protein kinase (AMPK), AICAR might play a key role in the regulation of energy homeostasis, increasing mitochondrial biogenesis without altering mitochondrial membrane potential.[10] Our data suggest that AICAR has the possibility to raise ATP production and counteract ROS production even in cases with likely impaired mtDNA biogenesis. If induction of mitochondrial biogenesis has a "therapeutic" role, or influences mechanisms fighting apoptotic cell death, or both will require further investigation.[15]

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Author contribution

Study concept and design: CeN, FMS. Acquisition of data: CeN, MCM, CaN, PG, LD, CV, RiC. Analysis and interpretation of data: CeN, PG, LD, AV, LV, FMS. Drafting the manuscript: CeN, FMS. Critical revision of the manuscript for important intellectual content: PG, LD, AV, LV. Study supervision: CeN, LV, FMS. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Founding

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Ethical approval

This study was approved by the Institutional ethics committee of the National Institute of Health, INSA, Porto, Portugal.

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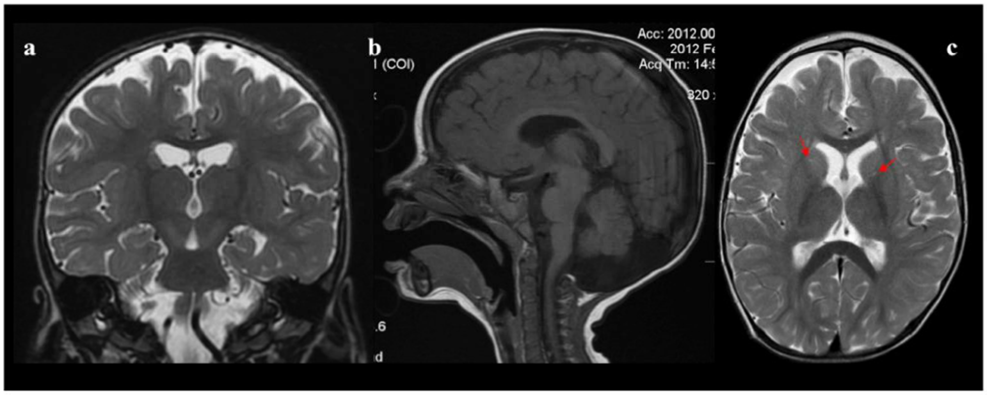
Legend to figures

Figure 1 – Brain MRI scan of the patient at the age of 18 months. Imaging disclosed mild cerebral atrophy and a slight hyopointensity of basal ganglia. Coronal (a), sagittal (b) and axial (c) scans are shown. Arrows indicate deep brain structures.

Figure 2 – Molecular analyses in a novel *SUCLA2* patient. **A**) Electropherograms of the *SUCLA2* gene flanking the homozygous c.985A>G/p.M329V in the index case (Pt). The wild-type sequence in a control (Ct) is also shown. Arrow indicates the mutated base. **B**) Prediction of functional effects of human variations using *in silico* analyses with PolyPhen-2, ClustalW aligment, and Sift prediction. **C**) Representative immunoblot analysis of fibroblasts homogenates from control (Ct) and patient (Pt) using specific antibodies against SUCLA2 and subunits of complex III (Core 2) and complex I (ND39). Porin was used to control for equal loading. The histogram shows the residual levels in the patient as compared to controls (n= 5) whose values were arbitrarily set as 100. Data represents the mean ± SD of three different determinations.

Figure 3 – Biochemical analyses in cells harboring the new c.985A>G/p.M329V in *SUCLA2*. **A**) Luminometric measurement of ATP expressed as relative luminescence units (RLU), in skin fibroblasts from controls (Ct) and patient (Pt) cultured in regular DMEM medium (RM) for 48 h. Cells were incubated 2 h in ATP record buffer supplemented with 10mM glucose (Glu), 2.5 µg/ml oligomycin (Oligo) and 5 mM 2-deoxy-Dglucose plus 5 mM pyruvate (2GD+Pyr). Data represents the mean ± SD of three different determinations. **B**) Reactive oxygen species (ROS) production measured by CM-H_2DCFDA and expressed as % of relative fluorescent units (RFU) in skin fibroblasts from controls (Ct) and the patient (Pt). Cells were cultured in medium supplemented with galactose (GAL) or Gal+5-Aminoimidazole-4-carboxamide ribotide (AICAR) 0.5mM, and

incubated with or without 0.5mM H_2O_2 for 30 min. Data are mean ± SD of three different determinations. Significance (*) was set at $p<0.05$.



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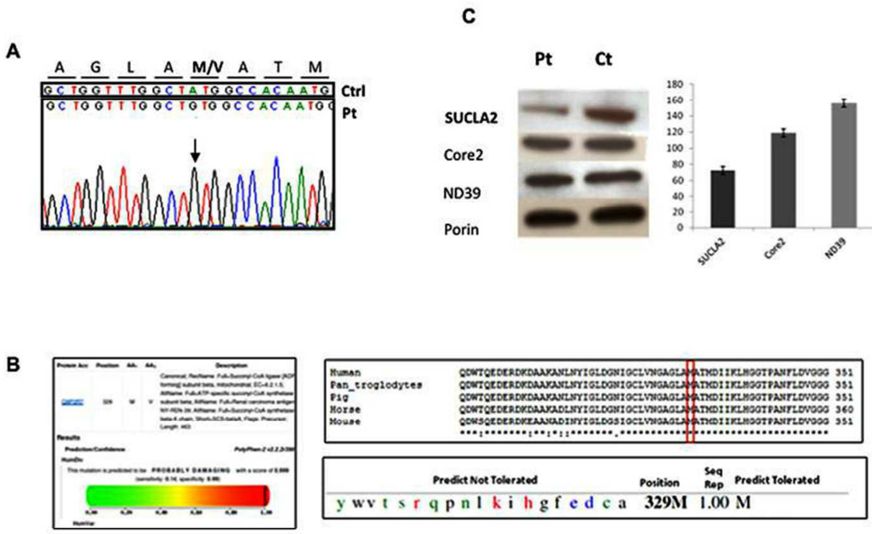

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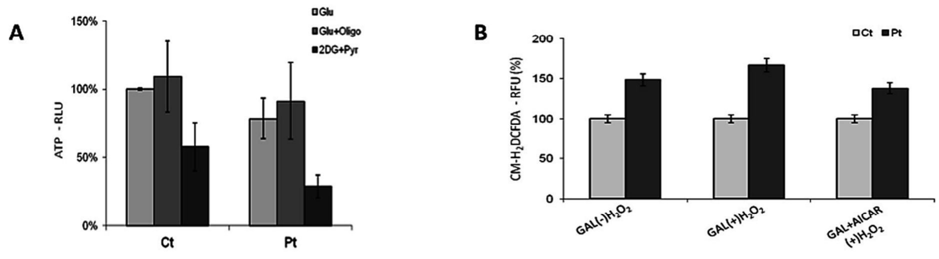

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Doenças da comunicação intergenómica: abordagem clínica e laboratorial

Doenças da comunicação intergenómica

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RESUMO:

As citopatias mitocondriais constituem um importante grupo de doenças metabólicas de expressão clínica heterogénea, para as quais não existe uma terapia eficaz. A maioria das doenças mitocondriais descritas são causadas por uma disfunção ao nível da fosforilação oxidativa (OXPHOS), originando consequentemente uma deficiente produção de energia. O correto funcionamento da OXPHOS resulta de uma interação coordenada entre o genoma nuclear e mitocondrial. Assim, as doenças mitocondriais podem ser causadas por defeitos moleculares no genoma mitocondrial, no nuclear, ou em ambos, originando doenças da comunicação intergenómica. Estas doenças resultam na perda ou na instabilidade do DNA mitocondrial (mtDNA), e podem causar quer deleções múltiplas, quer depleção do genoma mitocondrial.

As doenças da comunicação intergenómica adquiriram bastante relevância nos últimos anos tendo sido descritos um vasto número de genes associados a estas patologias, e podem ser divididas em dois grandes grupos: i) o síndrome das deleções múltiplas do mtDNA, que se caracteriza pelo aparecimento de miopatias oculares e dos membros e ii) o síndrome da depleção do mtDNA, que constitui um grupo de doenças autossómicas recessivas que se manifestam durante a infância ou início da adolescência com três apresentações clínicas distintas, nomeadamente miopática, encefalomiopática e hepatocerebral.

Neste trabalho pretendeu-se realizar uma revisão bibliográfica no sentido de apurar o "estado da arte" em relação às doenças de comunicação intergenómica. Serão abordados desde os fenótipos clínicos ao espetro mutacional e apresentado um algoritmo de diagnóstico, que possa ser uma ferramenta útil para os clínicos que se dedicam ao diagnóstico e *follow-up* deste tipo de patologias.

PALAVRAS-CHAVE: depleção do mtDNA, deleções múltiplas do mtDNA, doenças da comunicação intergenómica.

Nuclear-mitochondrial intergenomic communication disorders: clinical and laboratory approach

ABSTRACT:

Mitochondrial dysfunction accounts for an important and heterogeneous group of inherited metabolic disorders with hitherto no effective therapeutic options. Most of the known mitochondrial disorders are caused primarily by a dysfunctional oxidative phosphorylation (OXPHOS) and consequently a deficient energy production. OXPHOS depends on the coordinated expression of both nuclear and mitochondrial genomes. Therefore, mitochondrial diseases can be caused by genetic defects in the mitochondrial or the nuclear genome or in the interplay between the two genomes, causing nuclear-mitochondrial intergenomic communication disorders, that result in loss or instability of mtDNA leading to either qualitative (multiple deletions) or quantitative (depletion) lesions of mitochondrial genome. The mitonuclear crosstalk has gained increased relevance in the past years and since then many genes have been identified as being involved in these diseases. The problems faced by patients with mitochondrial cross-talk disorders, and mitochondrial disorders in general, are particularly severe. Multiple mtDNA deletion syndrome share features such as ocular and limb myopathy (PEO, ptosis, proximal weakness), which are almost always associated with extramuscular system involvement. From a clinical point of view mitochondrial DNA depletion syndrome (MDS) comprises a heterogeneous group of autosomal recessive disorders occurring in infancy or early childhood. It can be divided into three clinical categories: myopathic, encephalomyopathic and hepatocerebral MDS.

The focus of this review is to provide an overview of intergenomic communication disorders, a list of clinical phenotypes accompanied by their mutational spectrum and a diagnostic algorithm, which can be useful for clinicians when facing similar cases.

KEY-WORDS: Mitochondrial DNA depletion syndrome, Mitochondrial DNA multiple deletions, Mitochondrial diseases, Intergenomic communication disorders.

INTRODUÇÃO

Nos últimos 30 anos, um largo espectro de doenças multissistémicas associadas a disfunções da mitocôndria, designando-se globalmente de citopatias mitocondriais, têm sido referenciadas, com início dos sintomas desde o período neonatal até à idade adulta.[1] Estas disfunções podem afectar qualquer órgão ou tecido do organismo, embora os músculos esquelético e cardíaco, e o sistema nervoso central (SNC) sejam os mais afectados, devido à sua elevada dependência do metabolismo energético. Assim se compreende que as formas de apresentação mais frequentes incluam miopatia, encefalo(mio)patia e oftalmoplegia externa progressiva (PEO). Aproximadamente 1:5000 indivíduos na população adulta e infantil possuem doenças mitocondriais ou correm o risco de as virem a desenvolver.[2] Estes resultados revelam que estas doenças são uma causa comum de mortalidade e/ou morbilidade crónica, não estando disponível, até ao momento, nenhuma terapia eficaz.

Desde 1988 que a investigação molecular das citopatias mitocondriais tem sido focada no DNA mitocondrial (mtDNA) nomeadamente na pesquisa de rearranjos do mtDNA (deleções simples de diferentes tamanhos e duplicações) e pesquisa de mutações pontuais quer nos genes estruturais quer nos 22 tRNAs e dois RNAs.[3] Neste sentido, para aprofundar o conhecimento das citopatias mitocondriais em Portugal, implementou-se em 1993, na nossa Unidade, o estudo enzimático da cadeia respiratória mitocondrial (CRM) e posteriormente o estudo molecular do mtDNA.[4,5,6,7]

Nos últimos anos a investigação destas doenças tem-se centrado na pesquisa de mutações no DNA nuclear (nDNA), em genes que codificam subunidades estruturais da CRM / fosforilação oxidativa (OXPHOS), genes necessários à montagem das mesmas ou envolvidos na comunicação intergenómica.

As doenças da comunicação intergenómica tornaram-se num foco de estudo importante, dado que a grande maioria das proteínas mitocondriais é codificada pelo nDNA necessitando de serem importadas para a mitocôndria por mecanismos complexos, igualmente sob o controlo do genoma nuclear. Assim, neste trabalho efetuou-se uma revisão da literatura sobre este tema, focando os principais grupos de patologias, fenótipos clínicos e espetro mutacional.

MANIFESTAÇÕES CLÍNICAS E ETIOLOGIA MOLECULAR DAS PATOLOGIAS QUE AFETAM A INTEGRIDADE DO MTDNA

A integridade do mtDNA é mantida e controlada por um mecanismo complexo que envolve vários elementos do replissoma mitocondrial e diversas enzimas e transportadores que fornecem à mitocôndria os nucleótidos necessários (Fig. 1). Todos estes componentes são codificados pelo nDNA, logo mutações nos genes envolvidos na replicação e manutenção do mtDNA podem comprometer a sua integridade[8] causando quer deleções múltiplas quer depleção do mtDNA.[9] Nos últimos anos tem sido dada muito relevância à comunicação entre os dois genomas (comunicação intergenómica) e muitos genes têm sido identificados como estando envolvidos neste grupo de citopatias mitocondriais.

A tabela 1 sumariza as principais manifestações clínicas associadas ao síndrome das deleções múltiplas e da depleção do mtDNA (MDS), assim como a etiologia molecular relacionada com cada um deles.

1. Síndrome das deleções múltiplas do mtDNA

As doenças mitocondriais associadas à presença de deleções múltiplas do mtDNA ocorrem mais frequentemente em adultos e podem apresentar, na sua maioria, um padrão de hereditariedade autossómica recessivo (AR) ou dominante (AD), sendo este último mais frequente.[10] O tamanho das diferentes deleções do mtDNA é variável, até mesmo em doentes da mesma família. O espetro mutacional do síndrome das deleções múltiplas do mtDNA tem vindo a aumentar devido à descoberta de cada vez mais genes associados à instabilidade do mtDNA. Seguidamente será efetuada uma breve descrição das principais manifestações clínicas e as etiologias moleculares associadas.

i) PEO (Oftalmoplegia Externa Progressiva autossómica dominante ou recessiva)

As características clínicas mais comuns da PEO no adulto incluem a fraqueza dos músculos oculares externos, ptose bilateral, fraqueza muscular proximal e intolerância ao exercício. Sintomas como a presença de cataratas, perda de audição, neuropatia axonal sensorial, ataxia, depressão, hipogonadismo, e parkinsonismo também podem ocorrer. Características menos comuns incluem prolapso da válvula mitral, cardiomiopatia e alterações da motilidade gastrointestinal. Os indivíduos afetados apresentam deleções múltiplas ao nível do mtDNA que se encontram exclusivamente nos tecidos musculares.

Mutações nos genes *POLG*, *POLG2*, *C10orf2*, *SLC25A4*, *TK2*, *RRM2B*, *DGUOK*, *MGME1* *OPA1* e *MFN2*, encontram-se associados a PEO podendo apresentar uma hereditariedade AR ou AD sendo a forma AR geralmente mais grave.[10,11]

Seguidamente são referidos os principais genes envolvidos na etiologia molecular de PEO associados ao síndrome das deleções múltiplas.

POLG**:** a mitocôndria contêm uma única DNA polimerase, a polimerase gama (POLγ), imprescindível na embriogénese desde muito cedo.[12] A POLγ é codificada pelo nDNA sendo a única polimerase exclusivamente responsável pela replicação e reparação do mtDNA e essencial na sua manutenção. A POLγ é composta por uma subunidade catalítica , POLγA , que possui atividade de polimerase e de exonuclease; e uma subunidade acessória, POLγB, que aumenta a ação da enzima.[13] A POLγ juntamente com o mtDNA, a helicase do mtDNA e as diversas proteínas de ligação formam o complexo de replicação funcionante.[14]

As mutações no mtDNA eram consideradas a principal causa de doenças mitocondriais principalmente nos adultos, no entanto tem-se vindo a verificar que as patologias mitocondriais estão também associados a uma deficiente replicação do mtDNA. As mutações que se encontram na POLγA são uma das principais causas da doença mitocondrial podendo associar-se a uma grande variedade de sintomas clínicos, para além de PEO, tais como, Parkinsonismo, Sindrome de *Alpers - Huttenlocher*, encefalomiopatia neurogastrointestinal mitocondrial (MNGIE), neuropatia, ataxia sensorial, disartria e oftalmoparesia (SANDO) e ataxia espinocerebelar e epilepsia (SCAE).[15,16,17]

O gene *POLG* está localizado no cromossoma 15 e é composto por 23 exões que se extendem ao longo de 18,55 Kb. O gene foi identificado em 1996[18], mas somente em 2001 foi descrita a primeira mutação patogénica.[19] Desde então, mais de 200 mutações foram publicadas sendo este gene um *hot-spot* para mutações em doenças mitocondriais (Human Gene Mutation Database- HGMD, *POLG* database).[20] Mutações neste gene podem apresentar uma hereditariedade AD ou AR ou até a mesma mutação pode apresentar ambos os padrões de hereditariedade.[21,22]

Na forma AD de PEO causada por mutações no gene *POLG* (mais frequente), os doentes apresentam geralmente disfagia grave e disfonia e ocasionalmente, Parkinsonismo e disfunção cerebelar. Até à data mais de 65 mutações foram descritas no gene *POLG* como estando associado com a PEO (HGMD). No entanto, mutações neste gene podem também ser associadas à forma hepatocerebral de MDS.[15]

POLG2**:** a POLG2, a subunidade acessória da DNA polimerase gama, aumenta a eficiência da enzima. Alterações nesta subunidade podem causar acumulação de deleções do mtDNA. O gene *POLG2,* localizado no cromossoma 17, é composto por oito exões que se extendem ao longo de 19,28 Kb. Em 2006, a primeira mutação patogénica foi descrita como estando associada à forma AD de PEO.[23] No entanto, estão descritas mais 12 mutações neste gene associadas a fenótipos mitocondriais inespecíficos (HGMD).

***C10orf2* (*Twinkle*):** a helicase/primase mitocondrial codificada pelo gene *C10orf2* também é responsável pela forma AD de PEO.[24] Mutações neste gene estão associadas a apresentações clínicas que podem apresentar PEO "puro", ou uma forma mais complexa de PEO associada a fraqueza muscular proximal dos membros e músculos faciais, disfagia e disfonia, ataxia, e neuropatia periférica.

O gene *C10orf2* está localizado no cromossoma 10, sendo composto por 5 exões ao longo de 6,38 kb. A primeira mutação patogénica, associada com PEO, foi descrita em 2001[24] e desde então mais de 40 mutações patogénicas foram descritas, das quais 39 associadas a PEO (HGMD). Também foram descritas mutações associadas à forma hepatocerebral de MDS.[25,26]

***SLC25A4* (*ANT1*):** este gene codifica o transportador mitocondrial de adenina específico do músculo cardíaco (ANT1). A proteína ANT1 é a proteína mitocondrial mais abundante e, no seu estado funcional, é um homodímero de subunidades de 30 kD incorporados assimetricamente na membrana mitocondrial interna. O dímero forma um poro através do qual o ADP é transportado da matriz para o citoplasma. Nos mamíferos podem distinguir-se quatro isoformas específicas em diferentes tecidos.

O gene *SLC25A4* está localizado no cromossoma 4 e possui 4 exões distribuídos ao longo de 4,04 Kb. As primeiras mutações patogénicas foram descritas em 2000[27] e, desde então, apenas nove mutações estão referenciadas. Cinco destas estão associadas a AD de PEO, cujos doentes apresentam miopatia progressiva lenta com poucos ou nenhuns sintomas extra-musculares, três estão associadas a miopatia e a cardiomiopatia hipertrófica e a restante mutação descrita foi identificada num doente com SANDO.

***MGME1*:** o gene *MGME1* foi recentemente descrito[28] e localiza-se no cromossoma 20. É composto por quatro exões e codifica uma exonuclease mitocondrial que é essencial para uma síntese eficaz do mtDNA. É a primeira exonuclease mitocondrial identificada a estar envolvida na replicação do mtDNA, podendo ter uma função adicional de reparação, através do reposicionamento da cadeia de DNA ou das estruturas de DNA-RNA durante a síntese do mtDNA.

Encontram-se descritas duas mutações em três famílias distintas com PEO, que apresentam perda de peso severa e insuficiência respiratória.[28] As biópsias musculares dos doentes identificados revelaram quer deleções múltiplas, quer depleção do mtDNA.

ii) SANDO (neuropatia, ataxia sensorial, disartria e oftalmoparesia)

SANDO é uma doença sistémica, autossómica recessiva, caracterizada principalmente pelo aparecimento de neuropatia, ataxia sensorial, disartria e oftalmoparésia em adultos. O fenótipo é muito variável, mesmo dentro da mesma família, e pode ainda incluir miopatia, convulsões e perda de audição.[29] Foram descritas 14 mutações no gene *POLG* associadas a este fenótipo (HGMD).[16]

iii) SCAE (epilepsia e ataxia espinocerebelar)

SCAE é uma patologia semelhante a SANDO mas com uma maior frequência de enxaquecas e convulsões.[30] Estão descritas quatro mutações no gene *C10orf2* e uma no gene *POLG* associadas a esta doença.

iv) MNGIE (encefalomiopatia neurogastrointestinal mitocondrial)

MNGIE é uma doença AR com início entre a segunda e quinta décadas de vida caracterizada clinicamente por PEO, (pseudo)dismotilidade gastrointestinal, caquexia, leucoencefalopatia difusa, neuropatia periférica e morte precoce. Ao nível do mtDNA podem ser observadas deleções múltiplas e/ou depleção do mtDNA.[31] Mutações nos genes *TYMP* e *RRM2B* e mais recentemente no gene *POLG*[32] foram associados a este fenótipo. Seguidamente é referido o principal gene envolvido na etiologia molecular de MNGIE.

***TYMP (ECGF1)*:** o gene *TYMP* codifica a enzima timidina fosforilase (TP), que está envolvida no catabolismo das pirimidinas. Défices da enzima TP levam a uma acumulação sistémica de timidina e deoxiuridina, o que leva a um desequilíbrio da *pool* de deoxinucleótidos e consequentemente a uma instabilidade do mtDNA, originando o aparecimento quer de deleções múltiplas quer de depleção do mtDNA muscular.[33]
O gene *TYMP* está localizado no cromossoma 22 e é composto por 10 exões que se estendem ao longo de 4,3 kb. As primeiras mutações patogénicas foram descritas por Nishino e colaboradores[33] e, desde então, 85 mutações associadas a este fenótipo foram publicadas, a maioria no gene *TYMP* e as restantes distribuídas pelos genes *RRM2B* e *POLG.*

2. Síndrome da depleção do mtDNA (MDS)

O MDS caracteriza-se por uma redução acentuada do número de cópias do mtDNA e constitui um grupo de doenças raras e devastadoras que se manifestam maioritariamente logo após o nascimento, causando a morte prematura de muitos doentes durante a infância ou início da adolescência. Este síndrome é transmitido de modo AR, sendo genética e fenotipicamente heterogéneo e podendo apresentar-se sob três formas: miopática, encefalomiopática e hepatocerebral. Embora a maioria destas manifestações ocorram em tecidos específicos, afetando fundamentalmente o fígado, o músculo ou o cérebro, outros tecidos podem também ficar comprometidos com a evolução da doença.[34] O seu mecanismo fisiopatológico está relacionado com a manutenção da *pool* de nucleótidos mitocondriais, que assume um papel crucial na replicação e integridade do mtDNA.
Um défice múltiplo da CRM, e ainda mais crucial, o baixo *rácio* de mtDNA/nDNA nos tecidos afetados, ou seja, a depleção do mtDNA (superior a 65%), confirma o diagnóstico clínico deste síndrome.

Seguidamente é efetuada uma breve descrição das principais formas de MDS, assim como dos genes envolvidos na sua etiologia molecular.

i) Forma Hepatocerebral

A forma hepatocerebral é a apresentação mais comum de MDS. O aparecimento ocorre na infância (entre o nascimento e os 6 meses de vida) e os sintomas iniciais incluem vómitos persistentes, atraso de crescimento, hipotonia e hipoglicemia. As mitocôndrias do fígado apresentam normalmente, uma deficiência combinada dos complexos da CRM codificados pelo mtDNA. A morte ocorre normalmente dentro do primeiro ano de vida. Uma apresentação peculiar da forma hepatocerebral é o síndrome de *Alpers-Huttenlocher*, de aparecimento precoce e fatal, caracterizado por falência hepática, convulsões, evoluindo para epilepsia parcial contínua e deterioração neurológica global. A disfunção hepática é normalmente progressiva e evolui de esteatose microvesicular com proliferação dos ductos biliares para cirrose e falência hepática crónica. O uso de ácido valpróico como terapia para a epilepsia pode precipitar a falência hepática aguda. Mutações no gene *POLG* são uma causa frequente do síndrome de *Alpers-Huttenlocher*. Até ao momento, estão descritas cerca de 55 mutações associadas à forma hepatocerebral, distribuídas pelos seguintes genes: *DGUOK*, *MPV17*, *POLG*, *C10orf2*, *TK2* e *SUCLG1*. Genes, como *DGUOK* e *MPV17*, encontram-se também associados ao síndrome das deleções múltiplas do mtDNA.[35,36] Uma breve descrição destes genes será efetuada, com exceção dos genes *POLG e C10orf2* que foram descritos na secção anterior e dos genes *TK2* e *SUCLG1* que serão descritos seguidamente nas formas miopática e encefalomiopática de MDS, respetivamente.

***DGUOK*:** a cinase deoxiguanosina (DGUOK) fosforila a deoxiguanosina e a deoxiadenosina, contribuindo para a síntese de dois nucleotídeos necessários à manutenção da *pool* de desoxirribonucleótidos trifosfato (dNTPs) mitocondrial.[22,37]A associação de MDS com mutações neste gene sugere que a manutenção desta *pool* de dNTPs apresenta um papel crucial na replicação e integridade do mtDNA e consequentemente no seu conteúdo. Os doentes com mutações no gene *DGUOK*, localizado no *locus* 2p13, apresentam geralmente alterações hepáticas progressivas e dificuldades alimentares, associadas a disfunção neurológica (hipotonia, *nistagmus* e atraso psicomotor) por volta dos 3 meses de idade. A presença de neuropatia periférica e tubulopatia são raras mas, no entanto, foram descritas ocasionalmente.[38] A depleção do mtDNA está presente maioritariamente no fígado, não se observando no músculo nem nos fibroblastos. A nível histológico pode-se encontrar esteatose microvacuolar, colestase, fibrose e cirrose. Muitos casos apresentam uma evolução rápida e progressiva da doença, podendo a morte ocorrer até aos 12 meses de idade.[39] As primeiras mutações patogénicas descritas neste gene foram publicadas em 2001 por Mandel e colaboradores.[40] Atualmente, foram descritas mais de 50 mutações em cerca de 100 doentes (HGMD).

***MPV17*:** outro gene envolvido nesta forma clínica é o *MPV17* que se localiza no *locus* 2p23-p21 e codifica uma pequena proteína da membrana mitocondrial interna de função ainda pouco esclarecida. Estima-se que participa na manutenção da *pool* de dNTPs necessária para a síntese de mtDNA. O seu papel na patogénese do MDS é ainda desconhecido. Clinicamente estes doentes apresentam falência hepática severa, hipoglicemia, sintomas neurológicos e múltiplas lesões a nível cerebral durante o primeiro ano de vida.[41] A presença de grande quantidade de mtDNA depletado no fígado e/ou no músculo, assim como um défice múltiplo dos complexos da CRM, estão normalmente presentes nestes doentes. A nível histológico pode-se observar esteatose microvesicular e fibrose pericelular focal e periportal. Foram descritos aproximadamente 30 indivíduos com mutações no gene *MPV17*.[42,43] Desde a descoberta da primeira mutação neste gene [44], foram descritas mais de 25 mutações associadas a esta forma clínica e também à neuro-hepatopatia de *Navajo*, uma doença multissistémica autossómica recessiva encontrada na comunidade de *Navajo* no sudoeste dos Estados Unidos.[22]

ii) Forma miopática

Na forma miopática de MDS, o aparecimento de sintomas ocorre normalmente no primeiro ano de vida, com dificuldades alimentares, atraso de crescimento, hipotonia e fraqueza muscular. A creatina cinase (CK) está frequentemente aumentada, sendo um marcador importante para o diagnóstico, uma vez que não é muito comum em doentes com outras miopatias mitocondriais.[9] A morte ocorre normalmente nos primeiros anos de vida devido a insuficiência pulmonar e infeções, mas existem casos de alguns pacientes estudados que sobreviveram até à adolescência.[45] Estas manifestações clínicas e bioquímicas são acompanhadas de sinais morfológicos típicos de miopatia mitocondrial tal como a presença de fibras citocromo *c* oxidase negativas. A proliferação de mitocôndrias sob a forma de fibras rotas e vermelhas (RRFs) não é uma característica primária consistente mas, no entanto, pode aparecer mais tarde no decurso da doença. Defeitos bioquímicos em todos os complexos da CRM codificados pelo mtDNA estão sempre presentes nas mitocôndrias do tecido muscular.

***TK2*:** a timidina cinase (TK2) é uma enzima que fosforila a deoxitimidina, deoxicitosina e deoxiuridina, participando juntamente com a DGUOK na síntese de nucleotídeos necessários para a manutenção da *pool* de dNTPs mitocondrial.[46]

O gene *TK2* localiza-se no locus 16q22 e as mutações descritas neste gene estão especificamente associadas à forma miopática. Os sintomas aparecem durante a infância ou a adolescência e caracterizam-se por miopatia progressiva, culminando em falência respiratória e em morte, nos casos mais graves. No entanto os fenótipos menos graves apresentam uma evolução mais lenta da doença e um maior tempo de sobrevivência.[34]

Em 2001, Saada e colaboradores[47] identificaram pela primeira vez mutações neste gene. Depois desta primeira observação, um total de 37 mutações no gene *TK2* foram já descritas em mais de 50 doentes.[43] A maioria destas mutações estão associadas à forma miopática.

No entanto, existem mutações associadas a outros fenótipos, como é o caso da PEO, surdez neurosensorial e encefalopatia epilética (HGMD).

iii) Forma Encefalomiopática

Em 2005, Elpeleg e colaboradores[48], descreveram uma forma de encefalomiopatia autossómica recessiva associada a depleção do mtDNA. O aparecimento dos sintomas ocorre igualmente na infância, e manifestam-se através de hipotonia muscular, atraso psicomotor grave, deterioração neurológica progressiva, surdez, perda de movimentos voluntários, oftalmoplegia externa, convulsões generalizadas e variável disfunção tubular renal. Verifica-se um aumento do lactato sanguíneo e, na ressonância magnética cerebral, podem-se observar lesões nos gânglios basais sugestivas de síndrome de Leigh.[37]
Mutações nos genes, *RRM2B*, *SUCLA2* e *SUCLG1* estão associadas a esta forma clínica e encontram-se seguidamente descritas.

***RRM2B*:** o gene *RRM2B* localiza-se no locus 8q23 e codifica a subunidade R2 da reductase ribonucleotídica (RNR), uma enzima citosólica que está envolvida no passo terminal da síntese *de novo* dos dNTPs, convertendo os nucleotídeos em dNTPs durante a interfase do ciclo celular (fase S). A RNR está também envolvida no suplemento de dNTPs durante a reparação do DNA. Mutações neste gene estão associadas a hipotonia, acidose láctica, atraso de desenvolvimento e tubulopatia. A doença apresenta uma evolução rápida e fatal e manifesta-se frequentemente nos primeiros meses de vida. Verifica-se também a presença de grande quantidade de mtDNA depletado no músculo destes doentes.
Em 2007, Bourdon e colaboradores[49] descreveram a primeira mutação patogénica neste gene, estando atualmente mais de 30 mutações descritas em aproximadamente 15 doentes.[43]

***SUCLA2 e SUCLG1*:** a enzima succinil-CoA sintetase catalisa a síntese reversível do succinato e ATP a partir de succinil-CoA e ADP no ciclo dos ácidos tricarboxílicos (TCA). Esta enzima é constituída por duas subunidades, α e β, codificadas pelos genes *SUCLG1* (2p11) e *SUCLA2* (13q12), respetivamente. Mutações nestes genes parecem dificultar a associação entre a succinil-CoA sintetase e a cinase difosfato-nucleósido mitocondrial, resultando num desequilíbrio na *pool* de dNTPs mitocondrial e consequentemente na depleção do mtDNA no músculo.[50] Um dado importante para a suspeita do envolvimento destes genes é a presença de uma excreção urinária, moderada a elevada, de ácido metilmalónico e a presença de intermediários do TCA. Alguns doentes morrem durante a infância, no entanto outros apresentam uma maior sobrevivência. Clinicamente revelam hipotonia muscular proeminente, atraso psicomotor grave, distonia progressiva, surdez e convulsões generalizadas. Os doentes com mutações no gene *SUCLG1* podem ainda apresentar dismorfias pré-natais, crises metabólicas neonatais e a morte pode ocorrer nos primeiros meses de vida.

Estão descritos cerca de 30 doentes com mutações nestes genes, das quais nove no gene *SUCLA2*[51] e 13 no gene *SUCLG1* (*HGMD*).

ABORDAGENS DE DIAGNÓSTICO DAS DOENÇAS DE COMUNICAÇÃO INTERGENÓMICA

Nas doenças da comunicação intergenómica a apresentação clínica pode variar entre os síndromes bem definidos e um fenótipo multissistémico inespecífico onde o envolvimento neurológico está geralmente presente. Estabelecer um diagnóstico preciso num doente com suspeita de patologia mitocondrial revela-se um desafio, que requer uma abordagem multidisciplinar a nível clínico, bioquímico e histopatológico, para além de uma história familiar bem documentada. É importante obter as informações bioquímicas e/ou clínicas para uma investigação molecular adequada que permita identificar a mutação causal.

A determinação da atividade enzimática dos complexos enzimáticos da CRM é importante para orientar a abordagem molecular, em particular em doentes sem fenótipo específico. Como o mtDNA codifica subunidades de vários complexos da OXPHOS (CI, CIII , CIV e CV), é expetável que uma depleção do mtDNA provoque um défice combinado da OXPHOS (com exceção do CII, o único não codificado pelo mtDNA). No entanto a atividade enzimática dos complexos da CRM no músculo pode ser normal, se o tecido muscular não se encontrar entre os tecidos afetados, como acontece nos doentes com formas hepatocerebrais de MDS. A análise por *southern-blot* ou por PCR quantitativo em tempo real são dois dos métodos que detetam simultaneamente as deleções múltiplas e a depleção do mtDNA. Independentemente da tecnologia aplicada utiliza-se um gene nuclear de referência específico para a análise (*18S*) sendo muito importante o uso de controlos da mesma idade e do mesmo tecido, dada a natureza dinâmica da qualidade e quantidade do mtDNA.[52] Considera-se como *cut-off* para o diagnóstico de depleção do mtDNA uma redução do número de cópias de mtDNA superior a 65% em relação aos indivíduos controlo, podendo esta redução atingir os 80-90% em crianças, nos casos mais graves.

Os dados bioquímicos, tais como aumento de lactato e piruvato, quer no plasma no LCR ou na urina, alteração do perfil de ácidos orgânicos, aumento da alanina no perfil dos aminoácidos plasmáticos, assim como os dados de ressonância/tomografia cerebral são indicadores importantes para o diagnóstico destas patologias. Por exemplo num doente com mutações no gene *TK2* há um aumento da CK; mutações no gene *TYMP* estão associadas a um aumento de timidina no soro e urina; nos défices de SUCLA2 e SUCLG1 verifica-se um ligeiro aumento de ácido metilmalónico e de ácido metilcítrico na urina.[34]

O gene *POLG* é um *hot-spot* em casos de doença mitocondrial, e o seu estudo deve ser considerado em doentes que apresentem a sequenciação do mtDNA sem alterações e manifestem sinais clínicos inespecíficos tais como, hipotonia, atraso do desenvolvimento, epilepsia ou doença hepática. A toxicidade hepática induzida pelo ácido valproico em indivíduos com mutações nos genes *POLG* e *C10orf2* pode causar epilepsia fármaco-resistente.[53]

Com base na nossa experiência apresentamos um algoritmo para o estabelecimento de um diagnóstico para estas doenças da comunicação intergenómica (Fig. 2).

CONSIDERAÇÕES TERAPÊUTICAS

As citopatias mitocondriais não têm de momento qualquer terapia eficaz disponível, à exceção de um défice primário de ubiquinona (Q10) que são muito raros. O tratamento efectuado é meramente paliativo usando-se vitaminas, cofactores e substratos respiratórios que no entanto se revelaram pouco eficazes. Nos últimos anos, várias abordagens têm sido utilizadas e o desenvolvimento da biogénese mitocondrial (explicar melhor?) surgiu como uma possibilidade terapêutica.

No entanto, verifica-se que há uma estratégia diferente para cada caso. Por exemplo, o transplante hepático pode ser benéfico para doentes com hepatopatia causada por mutações no gene *DGUOK* que não tenham desenvolvido sintomas neurológicos, sendo a presença de hipotonia atraso psicomotor ou *nistagmus*, contraindicações para o transplante hepático.[54] Em doentes com mutações no gene *MPV17*, o transplante de fígado aumentou a qualidade e a expetativa de vida em alguns casos[55,56], embora desenvolvam sintomas neurológicos. Nestes doentes, foi sugerido a instituição de uma dieta controlada de modo a evitar a hipoglicemia e a protelar a insuficiência hepática.[57] Algumas crianças com mutações no gene *POLG* efetuaram um transplante hepático depois de insuficiência hepática induzida por ácido valproico, e embora o transplante tenha sido bem sucedido a evolução neurológica foi desfavorável.[58,59] Em alguns pacientes verificou-se uma melhoria da função hepática após tratamento com succinato ou coenzima Q_{10} ao qual se associou uma dieta rica em lipídios.[60] Nos doentes com MNGIE foi observada uma correlação entre os níveis plasmáticos da timidina e a gravidade do fenótipo[61]. Portanto, as tentativas para reduzir os níveis circulantes de nucleótidos, através da infusão de plaquetas de dadores saudáveis, podem resultar numa melhoria da doença, restaurando parcialmente a atividade da TP. No entanto a semi-vida curta das plaquetas é uma limitação desta terapia.[62] A diálise peritoneal tem sido usada para reduzir os níveis de timidina nestes doentes.[63]

Apesar de atualmente não existirem tratamentos totalmente eficazes para estas patologias, estão a ser identificadas novas terapias.[64]

CONSIDERAÇÕES FINAIS

A investigação em termos de diagnóstico neste grupo de patologias não é muito diferente da que se aplica noutras doenças e inclui a recolha de dados como história familiar, exame físico e neurológico, exames laboratoriais de rotina e específicos e eventual realização de biópsia muscular para estudo histopatológico, bioquímico e molecular.[65] Uma doença mitocondrial que se manifesta ao nascimento ou nos primeiros dias de vida é mais provável estar associada com alterações a nível do nDNA do que com alterações a nível do mtDNA. Os síndromes da depleção e de deleções múltiplas do mtDNA têm surgido cada vez mais como uma das principais causas de um amplo espetro de patologias multissistémicas de início na infância ou na idade adulta, respetivamente. Os avanços recentes na tecnologia de sequenciação, nomeadamente a utilização da sequenciação de nova geração, permitiram esclarecer a etiologia molecular em 55% dos doentes com suspeita de patologia mitocondrial, possibilitando a identificação de mutações causais em novos genes, por vezes não diretamente relacionados com patologias mitocondriais.[66,67,68]

A caraterização molecular destes doentes é importante não só para permitir a realização de aconselhamento genético e diagnóstico pré-natal adequados, nos casos em que forem identificados mutações no nDNA, mas também para melhorar a compreensão da fisiopatologia da doença. Uma estratégia de diagnóstico precisa e focada irá economizar recursos e possibilitará avanços fundamentais na compreensão da biologia mitocondrial, essencial ao desenvolvimento de novas estratégias terapêuticas.

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RECURSOS WEB

Human gene mutation database:

HGMD Professional database: www.hgmd.cf.ac.uk/

POLG mutation database:

http://tools.niehs.nih.gov/polg/index.cfm?do=polg.home&CFID=93263961&CFTOKEN=71342957

Tabela 1. Principais fenótipos clínicos e respetiva etiologia molecular associada às doenças de Comunicação Intergenómica (HGMD Professional database www.hgmd.cf.ac.uk/).

Fenótipos Clínicos	Genes	Número de mutações descritas	
Oftalmoplegia Progressiva Externa (PEO)	*POLG*	65	**Síndrome de delções múltiplas**
	POLG2	1	
	C10orf2	39	
	SLC25A4	5	
	*MGME1**	2	
	*TK2**	1	
	RRM2B	17	
	DGUOK	2	
Neuropatia, ataxia sensorial, disartria e oftalmoparésia (SANDO)	*POLG*	14	
	SLC25A4	1	
Epilepsia e ataxia espinocerebelar (SCAE)	*POLG*	1	
	C10orf2	4	
Cardiomiopatia hipertrófica	*SLC25A4*	3	
Encefalomiopatia neurogastrointestinal mitocondrial (MNGIE)	*TYMP**	81	
	RRM2B	2	**Síndrome da Depleção do mtDNA - MDS**
	*POLG**	1	
Síndrome da depleção do mtDNA - forma hepatocerebral	*POLG*	8	
	C10orf2	3	
	DGUOK	51	
	MPV17	28	
	TK2	1	
Síndrome da depleção do mtDNA - forma miopática	*POLG*	1	
	TK2	34	
	RRM2B	3	
	DGUOK	1	
Síndrome da depleção do mtDNA - forma encefalomiopática	*RRM2B*	14	
	TK2	1	
	SUCLA2	9	
	SUCLG1	13	
Síndrome de Alpers-Huttenlocher	*POLG*	54	

*Presença de deleções múltiplas e depleção do mtDNA

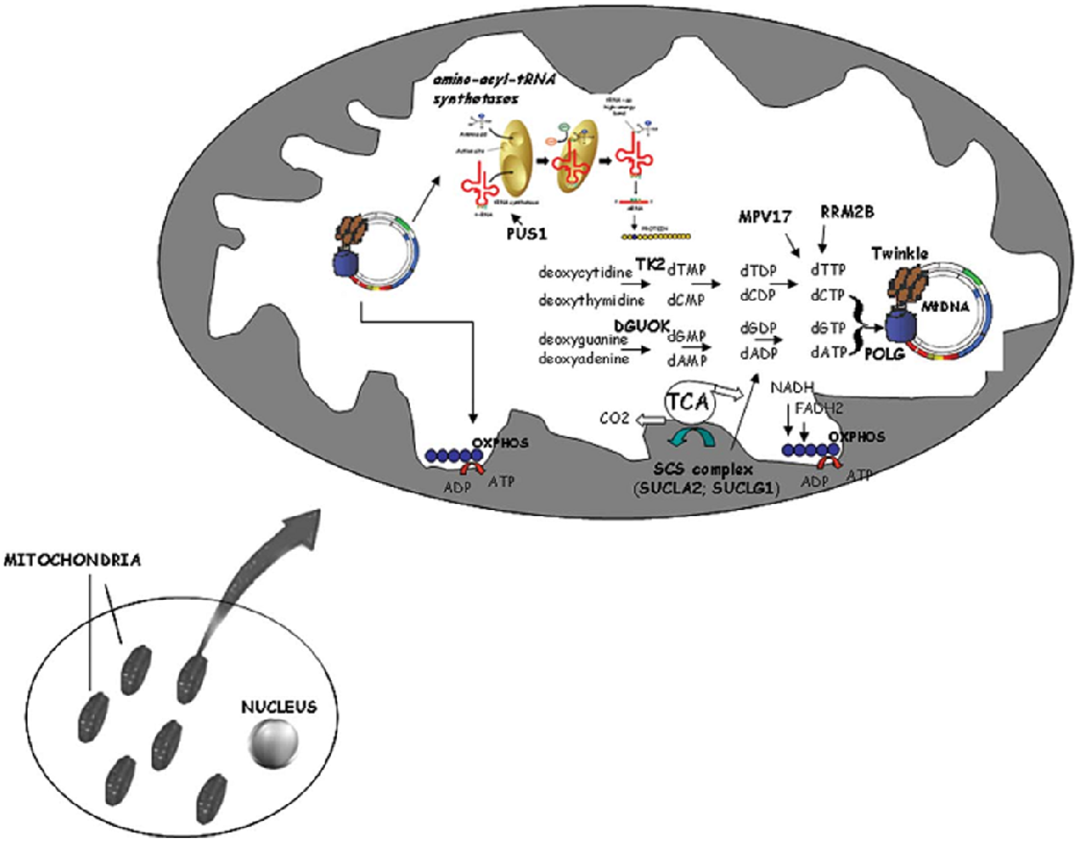

Fig. 1 Representação esquemática da mitocôndria e dos genes envolvidos nas doenças da comunicação intergenómica. Na mitocôndria podem-se identificar genes envolvidos na replicação do mtDNA (*POLG* e *C10orf2* - *Twinkle*), genes que afetam o metabolismo da *pool* de nucleótidos mitocondrial (*DGUOK*, *TK2*, *MPV17* e *RRM2B*), e genes envolvidos no ciclo dos ácidos tricarboxílicos (*SUCLA2* e *SUCLG1*) e que consequentemente afetam a fosforilação oxidativa. Estão também representados genes envolvidos na síntese proteica mitocondrial (*PUS1* e *aminoacil-tRNA sintetases*). Imagem de Nogueira e colaboradores.[7]

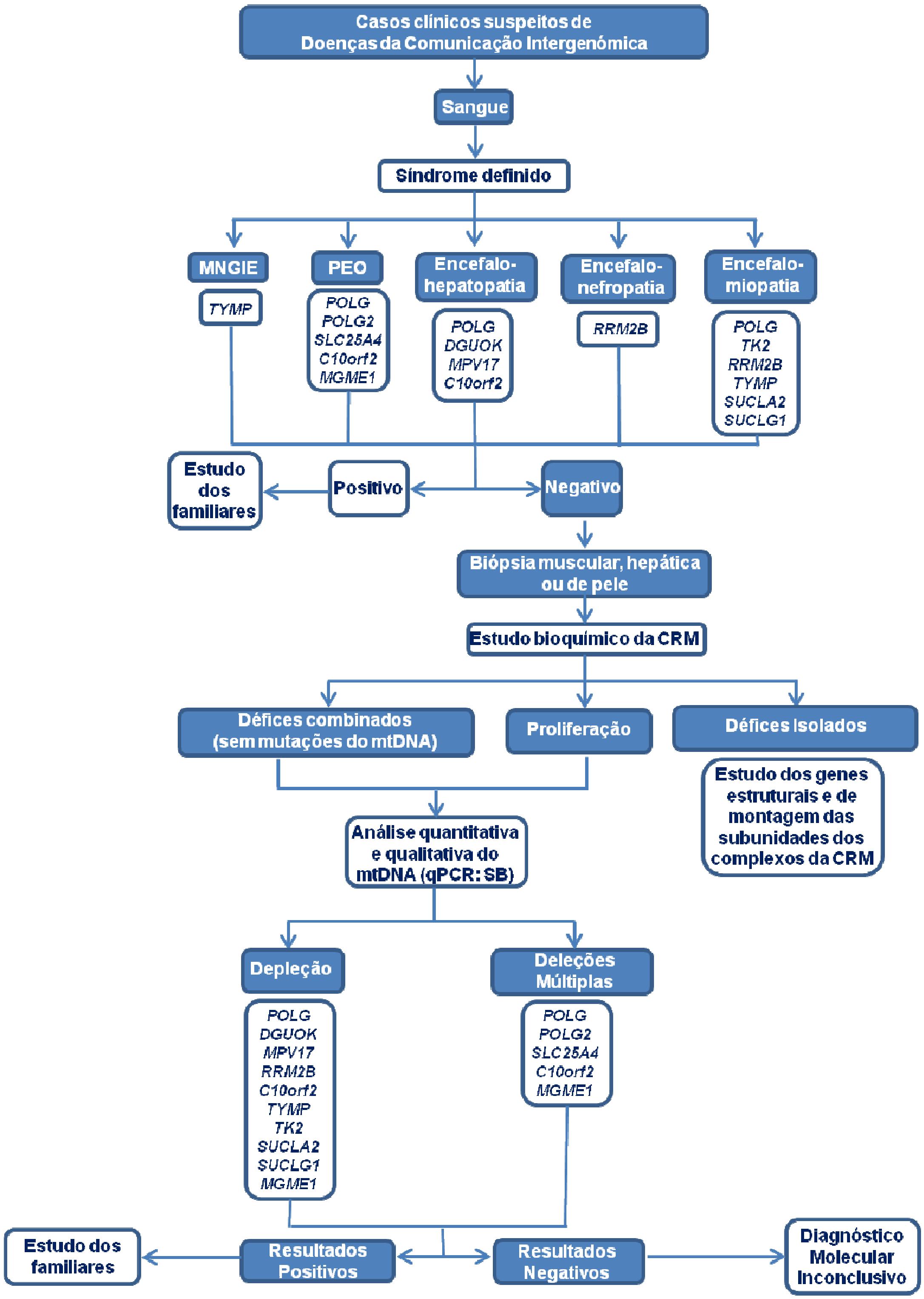

Fig. 2 Algoritmo de diagnóstico para as patologias da comunicação intergenómica, com base em dados clínicos e bioquímicos. CRM: cadeia respiratória mitocondrial; qPCR:PCR quantitativo em tempo real; SB: *Southern-Blot*.

The Syndromes Associated with Mitochondrial DNA Depletion: A critical review of a common disorder in the developmental age

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ABSTRACT

Mitochondrial dysfunction accounts for a relevant group of inherited metabolic disorders in large part caused by a dysfunctional mitochondrial respiratory chain (MRC) and consequently a deficient energy production. MRC function depends on the coordinated expression of both nuclear (nDNA) and mitochondrial (mtDNA) genomes. Thus, mitochondrial diseases can be caused by genetic defects in the mitochondrial or the nuclear genome or even in the interplay between the two genomes, causing the so termed nuclear-mitochondrial intergenomic communication disorders, that result in loss or instability of the mitochondrial genome leading to qualitative or quantitative lesions of mtDNA. In pediatric age, most MRC disorders are associated with nuclear gene defects rather than alterations in the mtDNA itself.

Mitochondrial DNA Depletion Syndromes (MDS) are a clinically heterogeneous group of autosomal recessive disorders occurring in infancy or early childhood, characterized by a reduced number of copies of mtDNA in the affected tissues and organs. At least four clinical categories of MDS are recognized: hepatocerebral, myopathic, encephalomyopathic and neurogastrointestinal. The focus of this review is to offer an overview of MDS, presenting a list of clinical phenotypes, their relative frequency, mutational spectrum, and possible pathogenic mechanisms involved in biogenesis and replication of mtDNA.

Keywords: Mitochondrial DNA Depletion Syndrome, mitochondrial myopathy, mitochondrial encephalomyopathy, hepatocerebral syndrome, mtDNA, OxPhos, Alpers-Huttenlocher syndrome.

INTRODUCTION

Mitochondria are dynamic cellular organelles, present in almost all eukaryotic cells, specifically involved in the production of cellular energy because of the mitochondrial respiratory chain (MRC) and the oxidative phosphorylation (OxPhos) system. In addition to the most important function, ATP production, mitochondria are involved in the regulation of other cellular pathways such as calcium homeostasis, apoptosis and programmed cell death [1].

Mitochondrial disorders are a group of genetically and phenotypically pleiomorphic disorders with an estimated incidence between 1:5,000 and 1:10,000 live births [2], they are often attributable to dysfunction of the OxPhos, which leads to a deficiency in ATP production.

The regulation of the MRC is the result of the interaction of two physically and functionally separate genomes: the nuclear DNA (nDNA) and the mitochondrial DNA (mtDNA) genomes. To carry out the OxPhos reactions mitochondria require the participation of about 1,500 genes encoded by both nuclear and mtDNA [3]. These genes encode for about 92 protein subunits forming the five multiprotein complexes embedded in the inner mitochondrial membrane. The mtDNA encode for 13 subunits of the OxPhos complex, for 2 ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) that are required for initiating translation and protein synthesis [4]. Therefore, although human mtDNA codes for the basic machinery of protein synthesis, a number of nuclear encoded factors are also needed, includingthe enzymes for replication, repair, and transcription, to carry out protein translation. This dependency lies at the heart of several newly recognized human diseases that are characterized by secondary abnormalities of mtDNA. The cross-talk between the two genomes is crucial for the maintenance of qualitative and quantitative mtDNA integrity and for correct mitochondrial protein production. Multiple deletions, depletion, or a combination of the two in critical tissues, are the hallmarks of disease conditions dysregulating the communication between the two genomes. A congruous amount of mtDNA is required for the production of key subunits of MRC complexes and therefore mtDNA depletion will result in organ dysfunction due to insufficient synthesis of respiratory chain components needed for adequate energy production [5].

MtDNA depletion syndromes (MDS) embrace a heterogeneous group of autosomal recessive disorders, characterized by low mtDNA levels in specific tissues. The syndromes are a consequence of defects in mtDNA maintenance caused by mutation in nuclear genes that function in either nucleotide synthesis (*TK2, SUCLA2, SUCLG1, RRM2B, DGUOK* and *TYMP*) or mtDNA replication (*POLG, C10orf2*). The first group of genes produce proteins that maintain the mitochondrial dNTP pool. dNTPs can be synthesized via either the *de novo* pathway (cell cycle-regulated) or the salvage pathway in which they are produced by utilizing preexisting deoxynucleosides to synthesize DNA precursors. Given that mtDNA replicates continuously and independently of cell division, mutations in any of the genes responsible for the dNTP pool will end in an impoverishment of the mitochondria from DNA building blocks will ensuing mtDNA depletion. On the other hand, mutations in *POLG* (the DNA polymerase gamma, the only DNA polymerase in humans that allows for replication and repair of mtDNA) and *C10orf2* (Twinkle, a helicase) result in impaired synthesis and incapacity to supply sufficient mtDNA to daughter cells during cell divisions resulting in reduction of low genome content [6].

MDS are rare, devastating diseases that manifest typically, although not exclusively, soon after birth, and lead usually to death in infancy or early childhood. MDS differ from more canonical MRC disorders, as they usually manifest as a tissue-specific disease [6,7]. However, it may occur that multiple organs, including the heart, brain, and kidneys, are affected [8].

The aim of this paper is to review the clinical manifestations and the molecular etiologies of nuclear defects involved in MDS and to provide possible insights towards best diagnostic strategies.

MDS – CLINICAL MANIFESTATIONS

MDS are usually classified as myopathic form (associated often with mutation in *TK2)*, encephalomyopathic form (associated with changes in *SUCLA2, SUCLG1,*and *RRM2B)*, hepatocerebral form (variants in *DGUOK, MPV17, POLG and C10orf2*) or neurogastrointestinal form (in *TYMP*) [9]. In Table 1 we summarized the main clinical manifestations and molecular etiologies associated with MDS.

Hepatocerebral MDS

The hepatocerebral MDS are probably the best known variants of the clinical condition and so far 55 mutations have been associatedwith this syndrome, including mutations in the *POLG*, *C10orf2*, *TK2*, *DGUOK*, *MPV17* and *SUCLG1* genes. The onset occurs within the first 6 months and death usually occurs within one year. Common symptoms include persistent vomiting, failure to thrive, hypotonia and hypoglycemia associated with progressive neurological deterioration. Histological changes on liver biopsy include fatty degeneration, bile duct proliferation, fibrosis and collapse of lobular architecture. Reduced cytochome c oxidase (COX) histochemistry and combined deficiency of mtDNA-encoded MRC complexes were detected in the liver of infants or toddlers . A peculiar form of hepatocerebral MDS is Alpers-Huttenlocher syndrome (AHS), an early-onset, fatal disease, characterized by hepatic failure, intractable seizures evolving into *epilepsia partialis continua*, and global neurological deterioration. The liver dysfunction is usually progressive as well, evolving from microvesicular steatosis with bile duct proliferation into cirrhosis and organ failure [10,11]. Brain MRI includes signal abnormalities in the basal ganglia and thalami, irregularly widened ventricles and partial pachygyria. Patients usually, but not always, present MRC deficiencies and low mtDNA in liver, although both may be normal in skeletal muscle. The clinical picture overlaps with hepatocerebral syndrome, and is probably among the severest diagnosis of infancy [12]. Mutations in *DGUOK*, *MPV17, POLG*, and *C10orf2* cause the hepatocerebral form of MDS.

i) DGUOK

Deoxyguanosine kinase (DGUOK) is a 2-deoxyribonucleoside enzyme that catalyzes the first step of the mitochondrial deoxypurine salvage pathway, namely, the phosphorylation of purine deoxyribonucleosides into the corresponding nucleotides deoxyguanosine and deoxyadenosine necessary for the maintenance of mitochondrial deoxynucleotide triphosphate (dNTP) pools [8, 13]. The typical phenotype of mutations in *DGUOK* is neonatal onset of progressive liver disease and feeding difficulties, usually associated with hypotonia, nystagmus, and psychomotor retardation by the age of 3 months. Most cases harbor null mutations and die before age 2 years. Peripheral neuropathy and renal tubulopathy have occasionally been reported [14]. Depletion of mtDNA has been documented mainly in the liver, where it results in combined reduction of complex I, III and IV; the amount of mtDNA is usually normal in other tissues, at least at disease onset. Histological analyses of liver biopsies show variable findings, including microvacuolar steatosis, and cholestasis. Progression is usually rapid with low life expectancy [15]. The majority of affected newborns show elevated serum concentration of tyrosine or phenylalanine on newborn screening. Intrahepatic cholestasis typically include elevation of liver transaminases, gammaglutamyltransferase and conjugated hyperbilirubinemia. An increased of serum concentration of ferritin is often observed [16,17].

Since the initial report of pathogenic mutations in 2001 [18], more than 100 affected patients have been reported and over 50 different *DGUOK* mutations have been identified (reviewed in [9]).

ii) MPV17

The *MPV17* gene lies on chromosome 2p23-p21 and encodes a mitochondrial inner membrane protein of unknown function. It has been suggested that MPV17 plays a role in controlling mtDNA maintenance and OxPhos activities in mammals and yeast [19]. The clinical presentation associated with mutations in *MPV17* consists of severe liver failure, hypoglycemia, growth retardation, neurological symptoms and multiple brain lesions during the first year of life [20]. Marked liver depletion associated with biochemical deficits are found, with complex I or I + III being the most affected. Both mildly reduced mtDNA content and defective OxPhos activities may also occur in muscle [21]. Histological analyses of the liver have revealed swollen granular hepatocytes, steatosis with focal pericellular and periportal fibrosis. Approximately 30 *MPV17*-related children have been reported [9]. At least 25 different mutations have been described in infantile-onset hepatocerebral syndrome and also in the allelic Navajo neurohepatopathy, an autosomal recessive multisystem disorder typical of the Navajo community in southwestern United States [13]. Three main subtypes are to be considered: infantile (onset < 6 months) and childhood (< 5 years) forms with hypoglycemic episodes and severe progressive liver dysfunction requiring liver transplant, and a “classic” form with moderate hepatopathy and progressive motor and sensory axonal neuropathy. The three subtypes are also associated with variable degrees of demyelination in both the central and the peripheral nervous systems.

iii) POLG

Human mitochondria contain a single DNA polymerase, polymerase gamma (POL γ), the only polymerase responsible for mtDNA replication and repair in mitochondria. POL γ is composed of a catalytic subunit that has both polymerase and proofreading exonuclease activities, and an accessory subunit, which increases enzyme processivity [17]. The holoenzyme functions in conjunction with the mtDNA helicase and the mitochondrial single-stranded DNA- binding protein to form the minimal replication apparatus [22].

The *POLG* gene on chromosome 15 comprises 23 exons spanning 18.55 kb. Over 200 mutations have been reported (Human DNA Polymerase Gamma Mutation database [23]) and *POLG* is considered a hot-spot for mutations associated with mitochondrial diseases [24, 25] with a large variety of clinical presentations, ranging from autosomal dominantly and recessively-inherited forms of progressive external ophthalmoplegia (PEO) to juvenile spinocerebellar ataxia and epilepsy (SCAE) with or without dysarthria, to AHS [12,26]. Approximately 45 different point mutations in *POLG* cause AHS [12] whose incidence has been estimated to be ~1:50,000 [27]. The two most common *POLG* mutations detected in AHS, i.e., A467T and W748S, can be either homozygous or heterozygous in combination with other variants. Carrier frequency for these mutations is higher in Western countries than elsewhere, For example, it is 1:125 for W748S in Finland; or 1:50 in Norway, if both variants are combined, whereas; 0.6% of normal Belgium population harbor the A467T, and a single ancestral founder mutations being hypothesized for both variants [6].

iv) *C10orf2* (*Twinkle*)

The mitochondrial protein Twinkle, encoded by *PEO1/C10orf2* (OMIM 606075) on chromosome 10q24, is a mtDNA helicase, active as a homohexamer and bound to mtDNA in mitochondrial nucleoids [28]. Mutations in *C10orf2* cause dominant disorders such as pure adult-onset PEO with multiple mtDNA deletions, or recessive clinical conditions including severe early-onset hepato-encephalopathy or infantile-onset spinocerebellar ataxia (IOSCA) and low mtDNA in brain and liver, but not in skeletal muscle [6]. Neuroimaging can show cerebellar cortical atrophy; OxPhos assays show reduced complexes I, III, and IV,.

IOSCA is a severe autosomal recessively-inherited neurodegenerative disorder that manifests after 9-18 months of age by progressive atrophy of the cerebellum, brain stem and spinal cord, ataxia during the first two years, hypotonia with sensory axonal neuropathy, optic atrophy, hearing impairment and ophthalmoplegia [6]. Patients usually survive to adult age. The severe neurological phenotype and the absences of muscle involvement observed in IOSCA suggest that Twinkle may play a crucial role in the maintenance and function of specific affected neuronal subpopulations [8].

IOSCA represents the second most common heritable ataxia in Finland because of the founder effect of the Y508C variant and a carrier frequency of about 1:200. The same mutation has also been described in cases with severe epileptict encephalopathy and hepatic failure [29].

Myopathic MDS

The symptoms of myopathic MDS usually appear in the first year of life and consist of feeding difficulties, failure to thrive, hypotonia, muscle weakness, and, occasionally, PEO. Death is often due to pulmonary insufficiency and recurrent infections, but some patients survive into their teens [30]. Muscle biopsy may show proliferation of mitochondria, and patchy or diffuse deficiency of COX. Biochemical defects of all mtDNA-related respiratory chain complexes are always present in muscle mitochondria. Serum creatine kinase (CK) levels may be variably elevated [31].

i) TK2

Thymidine kinase (TK2) is an intramitochondrial pyrimidine nucleoside kinase that phosphorylates dNTPs, such as deoxythymidine, deoxycytidine and deoxyuridine, thereby participating in the salvage pathway of deoxynucleotide synthesis [32]. Mitochondrial dNTP pools arise either through active transport of cytosolic dNTPs or through salvage pathways. Both pathways are essential for the replication of mtDNA, since the mitochondrion is unable to synthesize dNTPs *de novo*. Mutations in *TK2*, on chromosome 16q22, primarily affect muscle tissue, and have little or no effect on liver, brain, heart or skin. The clinical presentation of TK2-related MDS is variable, with a broad phenotype. Typical manifestation include a severe, rapidly progressing myopathy of infantile or childhood onset. Disease course is rapidly progressive, leading to respiratory failure and death in months or few years, although milder phenotypes with slower progression and longer survival have been described [6]. To date, about 50 individuals with *TK2*-related MDS have been reported [9]. Since description of the first mutation in 2001 [33], 31 different pathogenic autosomal recessive mutations have been described, and the different phenotypes may be explained by variable degrees of residual activity of the mutant enzymes. Mutations in *POLG* and *RRM2B* are additional etiologies in myopathic presentation of low mitochondrial copy number. Milder presentations manifest as late onset proximal muscle weakness, adult-onset progressive myopathy, with or without sensorineural hearing loss [9].

Encephalomyopathic MDS

Encephalomyopathic MDS are characterized by infantile onset of hypotonia with severe psychomotor retardation, high lactate levels in blood, progressive hyperkinetic-dystonic disorder, external ophthalmoplegia, deafness, generalized seizures and variable renal tubular dysfunction. Brain MRI is often affected and initially was suggestive of the pathological features seen in Leigh syndrome [8].

i) RRM2B

The *RRM2B* gene on chromosome 8q23 encodes the small subunit of p53-inducible ribonucleotide reductase, a heterotetrameric enzyme responsible for *de novo* conversion of ribonucleoside diphosphates into the corresponding deoxyribonucleoside diphosphates that are crucial for DNA synthesis [34]. The enzyme is the main regulator of the nucleotide pools in the cytoplasm and its small subunit is expressed in postmitotic cells, where it probably has a key function in maintaining the mitochondrial dNTP pools for mtDNA synthesis. Mutations in *RRM2B* usually result in neonatal hypotonia, lactic acidosis, failure to thrive and tubulopathy. Psychomotor delay, sensorineural hearing loss and a profound reduction of mtDNA copy numbers in skeletal muscle [34] are also present. The disease has a rapid progression and leads to death in a few months. The associated complex phenotype suggests that the consequences of defective mitochondrial dNTP pools can vary dramatically depending on the residual amount of functional enzyme. Approximately 15 infants have been described [9]. Of the 31 mutations thus far described, 30 are associated with major phenotypes.

ii) *SUCLA2* and *SUCLG1*

Succinyl CoA synthase is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinate and ATP or GTP from succinyl-CoA and ADP in the tricarboxylic acid cycle. This enzyme is made up of two subunits, *a* and *b*, encoded by *SUCLG1* on chromosome 2p11 and *SUCLA2* on 13q12, respectively. Mutations in *SUCLA2* and *SUCLG1* cause an encephalomyopathic form of infantile MDS, but *SUCLG1* can also cause a severe disorder characterized by antenatal dysmorphisms, neonatal metabolic crisis, and early death REFERENCE HERE. Differences in presentation between patients might depend on the lower residual amount of the protein [35-37]. Useful diagnostic clues in succinyl CoA synthase disorders are the presence of "mildly" elevated urinary methylmalonic acid in all patients and the presence of tricarboxylic acid cycle intermediates (methylcitrate, lactate, carnitine esters, 3-hydroxyisovalericacid) in most cases. Some patients die as infants (sudden infant death syndrome), but others show a longer survival. The clinical features include early childhood hypotonia, developmental delay and, almost invariably, progressive dystonia and sensorineural deafness. Mutations in *SUCLA2* and *SUCLG1* seem to disrupt the association between succinyl CoA synthase and mitochondrial nucleoside diphosphate kinase, resulting in an unbalanced mitochondrial dNTP pool and, eventually, in low mtDNA in muscle [38]. Thirty individuals, 20 with *SUCLA2* and 10 with *SUCLG1* mutations, have been reported (reviewed in [9]), with a total of 22 mutations, 17 of which are associated with encephalomyopathic and one with hepatocerebral MDS.

Neurogastrointestinal MDS

The MNGIE (mitochondrial neurogastrointestinal encephalomyopathy) syndrome is an autosomal recessive disorder clinically characterized by onset between the first and fifth decades with the vast majority of cases starting with symptoms in juvenile period before age 20 years. All affected individuals develop weight loss and progressive gastrointestinal dysmotility manifesting as early satiety, nausea, dysphagia, gastroesophageal reflux, postprandial emesis, episodic abdominal pain with distention, and diarrhea. In addition, all affected individuals have motor and sensory demyelinating neuropathy that may be accompanied by axonal neuropathy in some cases. The neuropathy typically presents with distal weakness and paresthesias occurring in a symmetric stocking-glove distribution. Ptosis and ophthalmoplegia are common. Affected individuals can have elevated CSF protein and plasma lactate. Thymidine and deoxyuridine are increased in plasma. Thymidine phosphorylase (TP) enzyme activity in leukocytes is usually less than 10 % of the control mean. Neuroimaging typically demonstrates diffuse white matter changes [9].

In MNGIE, abnormalities of mtDNA can include depletion, multiple deletions and point mutations [39]. Mutations in *TYMP* and *RRM2B* have been linked to MNGIE, though recent varints in *POLG* have been described in MNGIE-like syndromes [40].

Skeletal muscle generally shows ragged-red fibers and defects in single or multiple OxPhos complexes, especially COX. However, MNGIE has been reported without skeletal muscle involvement at the morphological, enzymatic, or mtDNA content level. Life expectancy is reduced (ranging from 25–60 years) [9].

i) *TYMP*

The *TYMP* gene encodes the cytosolic enzyme TP, which catalyzes the conversion of thymidine to thymine and deoxyuridine to uracil, and is therefore essential for pyrimidine catabolism. Defects of TP cause systemic accumulation of thymidine and deoxyuridine; this leads to deoxynucleotide pool imbalance and mtDNA instability, resulting in the presence of multiple deletions and partial depletion of muscle mtDNA [41].

TYMP is located at chromosome 22 and comprises 10 exons spanning 4.3 kb DNA. The first pathogenic mutations were described in 1999 [41] and since then over 70 mutations have been described, most of all associated with MNGIE.

MDS - DIAGNOSTIC APPROACHES

Suspicion of MDS is usually based on clinical presentation, which may range from well-defined syndromes to non-specific multisystem phenotypes, where neurological involvement is usually present. Establishing a specific MDS diagnosis is challenging and requires the integration of clinical assessments, family history, biochemical testing and histopathological examination in affected tissues. It is important to obtain the appropriate biochemical and clinical information before starting any molecular investigations in order to increase the chances of a successful molecular diagnosis. Biochemical determination of MRC complexes is also important, but can be normal, if skeletal muscle is not among the affected tissues. Quantitative real-time PCR quantification of total mtDNA content in affected tissues, using a nuclear gene as reference, represents a prerequisite for correct interpretation of the amount of mtDNA, though it is important to select the appropriate age-matched control materials [42] considering the dynamic nature of mtDNA copies in different ages and tissues. A reduction in mtDNA copy number to 60-65% of average age-matched controls is the empirical cut-off level for a diagnosis of primary MDS, but the reduction may be greater (80-90%) especially in children. Biochemical data, such as lactate, pyruvate, alanine and organic acid profiles, as well as neuroimaging findings, are also important clues to propose a diagnosis. Serum CK is especially elevated when mutations occur in *TK2* mutations, serum thymidine is impaired in *TYMP*, and a mild elevation of urinary methylmalonic acid and methylcitrate occur in disorders linked to *SUCLA2* or *SUCLG1* [6]. Reaching a full molecular definition is also important in adopting the appropriate therapies: identification of changes in *POLG* and *C10orf2* should prompt consideration for the risk of valproate (VPA)-induced liver toxicity in toddlers with severe drug-resistant epilepsy [43].

MDS - THERAPEUTIC CONSIDERATIONS

The management of mitochondrial disease is largely supportive as no “magic pill” is available [44]. Palliative treatments with vitamins, cofactors and respiratory substrates have been used, but showed poor efficacy. In recent years several approaches have been adopted mostly through a modulation of the different pathways regulating mitochondrial biogenesis [25], but they have not yet being applied translationally. In the meantime, therapeutic possibilities have been adopted, though none has an evidence based efficacy.

For example, liver transplantation may be beneficial to patients with hepatopathy caused by *DGUOK* mutations if no neurological symptoms have developed. However, significant hypotonia, psychomotor retardation or nystagmus would be contraindications for transplantation [16]. In patients with *MPV17*,and in VPA-induced organ failure, transplantation has increased quality and expectation of life in some patients [5,45], but children may later develop neurological symptoms [46,47]. A controlled diet avoiding hypoglycemia has been proposed to slow down disease progression and for supportive care [48]. Some improvement was suggested with succinate or coenzyme-Q10 together with a lipid-rich diet [49]. Also folate levels may be deficient in the cerebrospinal fluid (CSF) of some patients and recognition of low folate in the CSF may prompt replacement therapy [50]. Levocarnitine, creatine monohydrate, coenzyme Q10, B vitamins, and antioxidants, such as alpha lipoic acid, vitamin E, and vitamin C, have been often used as pro-energetic supplements in mitochondrial disorders in general and in MDS in particular but longer follow-up is necessary to appraise whether dietary interventions should be recommended [44]. In MNGIE, correlation between plasma thymidine levels and the severity of the phenotype has been observed [51]. Therefore, attempts to reduce the circulating nucleotide levels could result in disease improvement. Enzyme replacement therapy has been used in MNGIE: infusions of platelets from healthy donors reduced circulating thymidine and deoxyuracile levels and partially restored TP activity. The limitation of this therapy is the short half-life of the platelets [52]. Allogeneic stem cell transfusions have been given to two patients with MNGIE [53,54] and although more experience is needed to illustrate the clinical benefit of this treatment, it opens up a therapeutic possibility for disorders of the nucleoside metabolism. Finally, continuous ambulatory peritoneal dialysis has also been used in MNGIE to reduce thymidine levels, and this treatment improved symptoms during a three-year follow-up [55].

CONCLUDING REMARKS

A mitochondrial disease manifesting at, or soon after, birth is more likely to be associated with nDNA than with mtDNA mutations [25] but until very recently our profound ignorance regarding the mechanisms underlying mitochondrial gene transcription and translation and the complex interaction between the “two genomes” has limited our diagnostic power.

MDS have become an increasingly important cause of a wide spectrum of infantile and childhood-onset tissue-specific and multisystem disorders and could result from any imbalance of the mitochondrial nucleotide pool available for mtDNA replication, as well as abnormalities in mitochondrial replication machinery [56]. Consistent with the different phenotypes, mtDNA depletion may affect a specific tissue (most commonly brain and muscle or liver) or multiple organs, including heart and kidney. More than 75% of MDS patients manifest a full-blown condition during the first year of life, and the disease is rapidly fatal in most [57,58]. Identifying the causative genes is important not only to allow adequate antenatal options, family planning and prenatal diagnosis, but also to improve understanding of the disease pathophysiology and, therefore, improve therapeutic options. Thus, the recent advances in the clinical use of next generation sequencing (NGS) technologies will likely facilitate the molecular diagnosis in forthcoming years [59-61]. Since NGS is becoming a feasible option in several mendelian disorders and inborn error of metabolism [62], it also holds the promise to identify a greater number of patients with mitochondrial disorders as well [63,64]. This would likely resolve some of the open issues emerging from the clinic practice, including difficult diagnosis, uncertain counseling and, hopefully, unpredictable prognoses. An accurate and focused diagnostic workup will also save health-related resources and family distress. Only by achieving a full understanding of the molecular basis of MDS we will gather insights towards novel and effective therapeutic strategies.

COMPETING INTERESTS

The authors declare no conflicts of interest for the present paper.

AUTHORS' CONTRIBUTIONS

All the authors have made substantial contributions to conception and design of the review. All the authors have been involved in drafting the manuscript and revising it critically. All authors read and approved the final manuscript.

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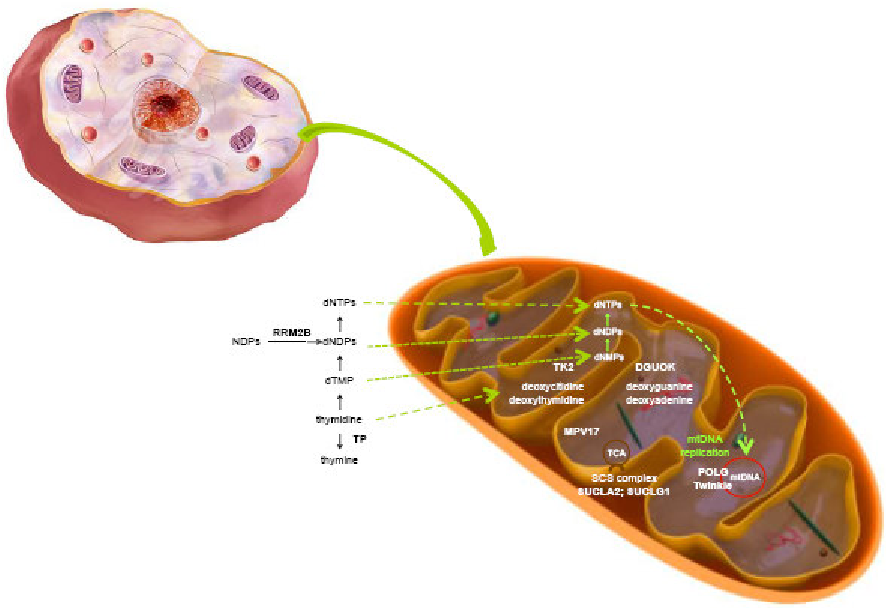

Figure 1. Schematic overview of the mitochondrion and the mitochondrial disease genes involved in MDS disorders. A “magnifying lens” focus on genes (namely, *POLG* and *C10orf2*-Twinkle) thought to be involved in replication of mitochondrial DNA (mtDNA), on those trusted important in the metabolism of the mitochondrial deoxynucleotide (dNTP) pool (via progressive phosphorylations of deoxythymidine, deoxycytidine, deoxytadenine, and deoxiguanosine); and on those belonging to the tricarboxylic acid cycle and secondary OxPhos involvement.

Table 1. Relative frequency of mutations associated with the different forms of Mitochondrial DNA Depletion (source: HGMD Professional database www.hgmd.cf.ac.uk/).

Clinical Manifestations	Genes	Number of mutations
Hepatocerebral mtDNA depletion syndrome	*POLG*	8
	C10orf2	3
	DGUOK	51
	MPV17	28
	TK2	1
Alpers-Huttenlocher syndrome	*POLG*	54
Myopathic mtDNA depletion syndrome	*POLG*	1
	TK2	34
	RRM2B	3
	DGUOK	1
	RRM2B	14
Encephalomyopathic mtDNA depletion syndrome	*TK2*	1
	SUCLA2	9
	SUCLG1	13
Mitochondrial Neurogastrointestinal Encephalomyopathy	*TYMP*	81
	RRM2B	2
	POLG	1